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# Intratumoral functional heterogeneity and chemotherapy

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## Abstract

Intratumoral heterogeneity including genetic and nongenetic mechanisms refers to biological differences amongst malignant cells originated within the same tumor. Both, cell differentiation hierarchy and stochasticity in gene expression and signaling pathways may result in phenotypic differences of cancer cells. Since a tumor consists of cancer cell clones that display distinct behaviours, changes in clonal proliferative behavior may also contribute to the phenotypic variability of tumor cells. There is a need to reveal molecular actions driving chemotherapeutic resistance in colon cancer cells. In general, it is widely hypothesized that therapeutic resistance in colorectal cancer is a consequence of the preferential survival of cancer stem cells. However, recent data regarding colorectal cancer suggest that resistance to anticancer therapy and post-therapeutic tumor reappearence could be related to variations of clonal dynamics. Understanding the interaction of genetic and nongenetic determinants influencing the functional diversity and therapy response of tumors should be a future direction for cancer research.



**Key words:** Colorectal cancer; Clonal dynamics; Functional heterogeneity; Chemotherapy; Xenograft; Oxaliplatin

**Core tip:** Beyond genetic diversity, a complex level of nongenetic mechanisms exists and drives the intratumoral inherent functional heterogeneity of tumor cells. Recent data suggest that changes in clonal dynamics of colorectal cancer cells can lead to drug resistance and tumor reappearance.

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## COMMENTARY ON HOT TOPICS

Cancer is a major worldwide health problem. It exists and grows due to uncontrolled proliferation of aberrant cells that are characterized by several different morphological and pathophysiological properties. This intratumoral cellular diversity remains a major challenge in our understanding both, the cancerous process and therapeutic resistance. Cellular heterogeneity can be resulted due to genetic and nongenetic mechanisms. However, the degree of interplay between these processes and their relative involvement in cancer propagation needs to be clarified.

Intratumoral heterogeneity, in part, arises through accumulated genetic changes that, within single tumors, result in several cellular subclones with significant biological differences[1-3]. On the basis of genetic changes, cell differentiation hierarchies can also contribute to cancer cell diversity[4-6]. Similarly, resistance to antitumoral therapies can arise on the basis of genetic mutations[7,8]. Nevertheless, the results of Kreso *et al*[9] indicate that biological differences amongst colorectal cancer cells can be provoked by the involvement of additional, nongenetic mechanisms.

## INTRATUMORAL GENETIC HETEROGENEITY

Cancer is the final result of successive genetic changes, disturbing regulatory processes and investing tumor cells with survival and growth advantages[10]. Genetic mutations lead to the selection of affected cells and their progeny[11]. Since intratumoral genetic heterogeneity is generally accompanied by variation in malignant behaviors[12], clonal genetic diversity of tumor cells has been correlated with poor prognosis[13]. In several types of cancers[3,14], heterogeneity in sequence mutations has been identified by exome sequencing of different regions of primary and metastatic tumors. These findings are of important clinical merit, given the current focus on using drugs that target specific mutant proteins and downstream signaling molecules.

## INTRATUMORAL NONGENETIC HETEROGENEITY

Besides genetic changes, some nongenetic (*e.g.*, epigenetic changes, posttranslational modifications, cell differentiation hierarchy) factors may also influence cell-cell variability within a tumor.

To explore functional equivalence of cancer cells within single genetic clones Kreso *et al*[9] combined deoxyribonucleic acid (DNA) copy number alteration (CNA) profiling, sequencing, and lentiviral lineage tracking, followed the repopulation dynamics of 150 single lentivirus-marked lineages from 10 human colorectal cancers over multiple serial transplantations in mouse xenografts.

DNA CNA profiling and mutational hotspot deep sequencing in 42 cancer-related genes indicated that a number of tumor xenografts preserved the genomic profile of the primary tumor, whilst in some cases substantial genetic differences were observed between the first transplant and the parental tumor, indicating the presence of clonal selection during xenograft growth. However, the latter cancer cells also remained genetically stable in subsequent transplants. Furthermore, the results of deep sequencing proved high concurrence amongst distinct single cell-derived clones. Based on these results, clonal stability of colorectal cancer cells seems to be maintained through serial tumor transplantations. Consistent with earlier results[15], Kreso *et al*[9] showed that xenografting itself did not select for a significantly different tumor cell population in relation of multiple recipients at each stage of serial transplantation.

In spite of the observed genetic homogeneity, the different, lentiviral marked cancer cell clones displayed different biological behaviours during serial transplantations. Persistent cell clones were present in all serial transplants. Short-term clones did not persist, but exhausted before reaching the final passage. Transient clones were only detected in the first recipient, and were not detected in the second and subsequent recipients, therefore these clones lacked tumor-propagating ability. The presence of these clones suggests that there is a substantial functional diversity with respect to clonal longevity in the course of successive tumor transplantations. Additionally, cell clones with different dynamic behaviour were also observed. Resting clones were likely produced by cancer cells that were initially dormant (or slowly proliferating), but became activated during later transplants, finally resulting in a measurable clone. Fluctuating clones consisted of cells whose progeny appeared early, but they became undetectable in a subsequent transplantation, and only to reappear later. These clones displayed intermittently extensive proliferative capacity. These results indicate that not all cancer cells having the potential for tumor propagation actually function and contribute to tumor growth at a given time. There are cells that can become activated at a later point of tumor progression. The distinct clonal proliferative kinetics observed by Kreso *et al*[9] underscore the functional variability of individual cells. Taking into account the absence of changes in CNAs and single nucleotide variants with serial transplantations, these data provide evidence for functional heterogeneity amongst individual tumor-propagating cells with a shared common genetic lineage. This phenotypic diversity results from the integration of both genetic and nongenetic influences. Nongenetic factors include stochasticity in gene expression, epigenetic regulation, and microenvironmental variability[16,17].

Based on the presence of functionally heterogenous cancer cell clones, Kreso *et al*[9] further investigated the response of these cells to oxaliplatin chemotherapy. Although the authors have found that chemotherapy reduced tumor mass, no apparent change in the absolute number of marked clones, or the proportions of clone types were observed. Tumor propagation capability of cancer cell clones was also found to be altered after oxaliplatin therapy. Despite eradication of some lentiviral marked (mainly persistent cell) clones, resting or slowly proliferating cancer cells endured oxaliplatin therapy and reinitiated tumor growth in a slower manner. The results of CNAs, single nucleotide variant and methylation pattern analyses indicated that oxaliplatin-treated cells genetically closely matched to the control recipients. These data suggest that therapeutic tolerance is not always caused by the acquisition of new driver mutations. Behalf, alterations of tumor propagation behaviour of individual cancer cells can act as a nongenetic determinant of tumor response to chemotherapy. Similarly, in human lung cancer a small subpopulation of "anti-epidermal growth factor receptor therapy-tolerant" cells were found[18]. This subpopulation of cells demonstrated a highly reduced drug sensitivity and maintained viability *via* engagement of insulin-like growth factor 1 receptor (IGF1R) signaling and an altered chromatin state that required histone demethylase activity. However, the drug-tolerant subpopulation could be selectively ablated by treatment with IGF1R-inhibitors or chromatin-modifying agents, potentially yielding a therapeutic opportunity.

Although Kreso *et al*[9] did not discover mechanisms responsible for the variability in clonal behavior, their study has several important values. It emphasizes the need

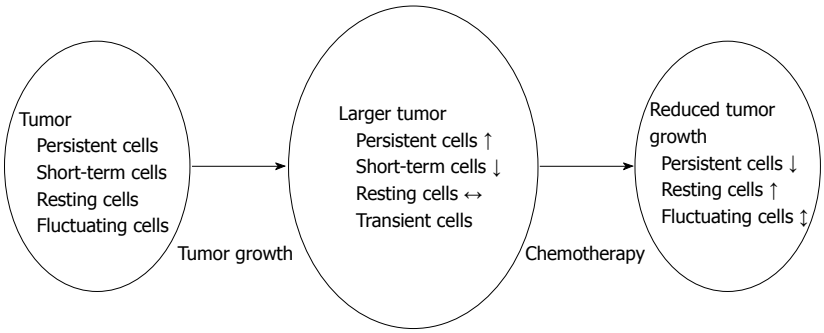


**Figure 1 Connection between clonal behaviour and cellular constitution of the tumor.** Differences in clonal behavior result in changes of the cellular constitution of colorectal cancer during normal tumor growth and after chemotherapy as well. In the case of unperturbed tumor growth the proportion of the persistent cell clone increases, short-term cells fade away, while the number of resting cells remains unchanged. Transient cells may appear but later they also fade away. After oxaliplatin therapy the number of the highly proliferative (hence chemotherapy sensitive) persistent cells significantly decreases, while the drug resistant resting cell clone contributes to tumor reappearance.

of adequate understanding of nongenetic processes that underlie phenotypic heterogeneity of cancer cells.

Upon technical procedures, a shift from classical assaying techniques using bulk cell populations (and masking single-cell level heterogeneity) to newly developed/advanced methods (*e.g.*, combining laser pressure catapulting techniques with bisulfite-based arrays, next-genome sequencing arrays or whole genome gene expression arrays) is expected. Moreover, their findings highlight on demand of efforts to reveal molecular actions driving chemotherapeutic resistance in colorectal cancer cells. In general, it is widely hypothesized that therapeutic resistance in cancer is a consequence of the preferential survival of cancer stem cells. However, the results of Kreso *et al*[9] suggest that cellular drug resistance and post-therapeutic tumor reappearence could not only be related to the stem-cell characteristics, but also to variations of clonal dynamics (Figure 1).

Recent findings of Kreso *et al*[9] reveal that, beyond genetic diversity, a complex level of nongenetic mechanisms exists and drives the intratumoral inherent functional heterogeneity of tumor cells. Thus, understanding the interaction of genetic and nongenetic determinants influencing the functional diversity and therapy response for cancers should be a prominent future direction for cancer research.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

# Sex hormones in the modulation of irritable bowel syndrome

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## Abstract

Compelling evidence indicates sex and gender differences in epidemiology, symptomatology, pathophysiology, and treatment outcome in irritable bowel syndrome (IBS). Based on the female predominance as well as the correlation between IBS symptoms and hormonal status, several models have been proposed to examine the role of sex hormones in gastrointestinal (GI) function including differences in GI symptoms expression in distinct phases of the menstrual cycle, in pre- and post-menopausal women, during pregnancy, hormonal treatment or after oophorectomy. Sex hormones may influence peripheral and central regulatory mechanisms of the brain-gut axis involved in the pathophysiology of IBS contributing to the alterations in visceral sensitivity, motility, intestinal barrier function, and immune activation of intestinal mucosa. Sex differences in stress response of the hypothalamic-pituitary-adrenal axis and autonomic nervous system, neuroimmune interactions triggered by stress, as well as estrogen interactions with serotonin and corticotropin-releasing factor signaling systems are being increasingly recognized. A concept of "microgenderome" related to the potential role of sex hormone modulation of the gut microbiota is also emerging. Significant differences between IBS female and male patients regarding symptomatology and comorbidity with other chronic pain syndromes and psychiatric disorders, together with differences in efficacy of serotonergic medications in IBS patients confirm the necessity for more sex-tailored therapeutic approach in this disorder.



**Key words:** Brain-gut axis; Irritable bowel syndrome; Microbiota; Pain modulation; Sex hormones

**Core tip:** Recent clinical and experimental findings support the modulatory actions of sex hormones exerted at different levels of the brain-gut-microbiota axis in irritable bowel syndrome (IBS). Sex hormones may influence peripheral and central regulatory mechanisms contributing to the alterations in visceral sensitivity, motility, permeability, and immune activation of intestinal mucosa. A new concept of "microgenderome" is emerging based on the observations that the gender bias present in numerous diseases may be reinforced by the commensal microbiota of the host. Significant sex differences in epidemiology, symptomatology, and treatment outcome in IBS indicate the necessity for sex-tailored therapeutic approach in this disorder.

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## INTRODUCTION

Sex hormones, in particular estrogens, play a significant role in the physiology and pathology of the gastrointestinal (GI) tract including regulation of motor and sensory function[1,2]. Irritable bowel syndrome (IBS) is a GI sensory-motor disorder characterized by abdominal pain or discomfort associated with a change in bowel habits[3]. The role of gonadal hormones in symptomatology and pathophysiology of IBS is being increasingly recognized based on the female predominance as well as the correlation between IBS symptoms and hormonal status during menstrual cycle phases, pregnancy or menopause[4]. Sex differences in stress and pain response are considered as crucial factors in the pathogenesis of functional GI disorders[4,5]. Sex hormones influence peripheral and central regulatory mechanisms of the brain-gut axis involved in the pathophysiology of IBS contributing to alterations in visceral sensitivity, motility, permeability, and immune activation of intestinal mucosa[4,6,7]. Among numerous interactions of sex hormones with other neurotransmitters, estrogen interactions with serotonin and corticotropin-releasing factor (CRF) signaling systems play a pivotal role[8,9]. Estrogens can also modulate neuroimmune interactions triggered by stress *via* the brain-gut axis[10]. Recently, the gut microbiota has been also recognized as an important element in the bi-directional communication along the brain-gut axis through neural, immune, and endocrine pathways[11,12]. In the present article we will review recent clinical and experimental findings supporting the modulatory effect of sex hormones, in particular estrogens, on different levels of the brain-gut-microbiota axis in IBS and their clinical implications regarding the symptomatology, pathophysiology and treatment of IBS.

## SEX AND GENDER DIFFERENCES IN IBS PREVALENCE AND SYMPTOMATOLOGY

In Western countries the female-to-male ratio among non-patient population of IBS sufferers is 2:1[13]. Within the patient population in primary or tertiary care settings females outnumber male patients by 3:1 to 5:1, respectively[13-15]. However, in many Eastern countries such as India, China and South Korea, the female predominance among IBS patients is not observed[16]. Likewise, results of recent meta-analysis studies in South Asia, South America, or Africa confirmed that IBS prevalence was not significantly higher in women, compared to men[17]. Therefore gender-related and socio-cultural differences in health care-seeking behavior are suggested to also account in IBS symptoms reporting[18]. Further epidemiologic studies from different world regions are needed to elucidate the complex interactions between genetic, environmental, psychological and/or cultural factors that may contribute to sex differences in IBS symptoms[19,20].

Additionally, as the prevalence of IBS subtypes varied according to gender[17], the dominating subtype of IBS in different countries may also affect the male-to-female ratio. Women with IBS, compared to male patients, are more likely to report constipation, bloating, severe abdominal pain, and feeling of incomplete evacuation, while men with IBS more frequently complain of diarrhea-associated symptoms[17,19,21]. In fact, earlier studies indicate that women have slower colonic transit in comparison with men[22,23]. The results of the recent study by Tang *et al*[24] conducted in Chinese population confirmed significant differences between female and male IBS patients in their rating of abdominal pain/discomfort with regard to severity and duration, but not frequency of pain attacks. Interestingly, sex differences in dietary coping with GI symptoms have also been reported[25]. Female IBS patients seem to be more willing to implement nutritional behavior changes alleviating the GI problems than men, although both men and women could benefit similarly from these changes[25].

Cain *et al*[26] reported higher GI symptoms (pain, distension, bloating, intestinal gas) in postmenopausal women than in men, but the greatest differences in the overall symptom reporting between men and women were associated with somatic symptoms such as joint and muscle pain. This gender-related difference was most prominent when postmenopausal women where compared to men. Gender differences were much weaker for psychological and emotional symptoms except for fatigue, sleep disturbances and stress[24,26]. Noteworthy, there is a wide spectrum of chronic pain disorders frequently overlapping with IBS namely fibromyalgia, migraine headache, chronic pelvic pain, interstitial cystitis, and chronic fatigue syndrome. These diseases are also characterized by female predominance with a correlation between their symptoms and hormonal status[27-29]. In addition, women with IBS, more frequently show comorbidity with affective or mood symptoms including anxiety and depression as compared to women without IBS[30]. There are also reports indicating that women with IBS exhibit more anxiety and depressive symptoms compared to men with IBS[24,26]. Sex differences in the prevalence of concomitant somatic symptoms, as well as anxiety and depression may significantly contribute to the greater impairment of quality of life in female patients and affect treatment results[24,31].

Sex-related difference in IBS prevalence emerges around the time of puberty and increases during the early adult years. Women are suffering from IBS most commonly in the late teenage to mid forties, additionally suggesting the role of reproductive hormones in the pathophysiology of the disorder. The incidence of IBS in women steadily declines with age and approaches the rate observed among men by the 7$^{th}$ decade of life[32]. By contrast, the prevalence of IBS among males is fairly constant within the age range of 20-70 years[32].

## CORRELATION BETWEEN IBS SYMPTOMS AND HORMONAL STATUS

### *Menstrual cycle*

The menstrual cycle in women is divided into three

**Table 1 Correlation between hormonal status and irritable bowel syndrome symptoms expression[36]**

| Status | Hormone levels | IBS and pain related symptoms expression | Ref. |
|---|---|---|---|
| Late luteal phase (premenses) | Rapid decline in estrogen and progesterone levels | Exacerbation of bowel symptoms<br>Increased bloating | [33,34] |
| Menstruation (menses) | Lowest levels of estrogen and progesterone | Exacerbation of bowel symptoms<br>Increased abdominal pain/discomfort<br>Lower rectal sensitivity threshold | [34,35,37,38,40] |
| Dysmenorrhea | Disturbances in hormonal interactions at different regulatory levels (lower progesterone level) | Exacerbation of bowel symptoms | [41] |
| Pregnancy | Physiological hyperestrogenemia and hyperprogesteronemia | Reduced pain sensitivity and alleviation of many chronic pain syndromes<br>Exacerbation of constipation (prolonged gastrointestinal transit) | [27,51,73] |
| Menopause | Decline in ovarian hormones | Decrease in IBS incidence<br>High prevalence of constipation and somatic discomfort syndromes | [26,53,54] |
| Oral contraceptives | Estrogen and progestin administration | Reduced abdominal symptoms at menses | [55] |
| Hormone replacement therapy | Estrogen (and progesterone) supplementation | Increased prevalence of IBS in postmenopausal women during HRT<br>Prolongation of IBS symptoms to a later age | [58] |
| Oophorectomy | Ovarian hormone deficiency | Exacerbation or occurrence of gastrointestinal symptoms after gynecological surgery | [60] |
| Men with IBS | Lower level of luteinizing hormone in middle-aged men<br>Elevated level of sex hormone-binding globulin in young men | Generally more prevalent diarrhea (compared to women with IBS) | [66,70] |
| Transsexual women (male-to-female subjects) | Estrogen/anti-androgen treatment | Development of chronic pain including visceral pain | [72] |

IBS: Irritable bowel syndrome; HRT: Hormone replacement therapy.

phases: the follicular (proliferative) phase, ovulation, and the luteal (secretory) phase. Estrogen levels are increasing during the midfollicular phase and then drop precipitously after ovulation. This is followed by a secondary rise in estrogen levels during the midluteal phase with a decrease before menstruation. The secondary rise in estradiol parallels the rise of serum progesterone and 17-hydroxyprogesterone levels[33].

Dynamic changes in ovarian hormones during menstrual cycle can modulate GI contractility, transit, secretion, visceral sensitivity, and immune function at multiple target sites, including those located in the periphery and the brain[3]. Clinical studies indicate that declining or low ovarian hormone levels in women (such as during menses) may contribute to the occurrence or exacerbation of GI symptoms, including abdominal pain or discomfort, altered bowel habits and bloating that varies across the menstrual cycle phases (Table 1)[34-37]. Rectal sensitivity thresholds have been shown to be significantly lower in IBS patients at menses relative to those at other cycle phases indicating that IBS symptoms experience may be modified by ovarian hormone status[38]. Also in animal studies it has been shown that both visceral and somatic sensitivity vary over the rat estrous cycle and that high levels of ovarian hormones (proestrus/estrus stages) are associated with enhanced sensitivity[39]. Therefore the menstrual cycle provides a natural model to explore the effects of ovarian hormones on the bowel function.

Approximately one third of otherwise asymptomatic women experience GI symptoms at the time of menstruation[34]. About 40% of women with IBS report influence of the menstrual cycle on their symptoms[35]. Whitehead *et al*[37] found that in women with functional bowel disorders (FBDs), including IBS, bowel symptoms seem to be affected by menstruation to a greater degree than in women without FBDs, suggesting that IBS women may respond differently to the fluctuations in the ovarian hormones. Variation in GI symptoms during the menstrual cycle can be related to motor disturbances and/or a change in perception of colonic motor events, as well as alterations in colonic epithelial barrier and mucosal immunity[10,40].

IBS female patients are more likely to report dysmenorrhea and premenstrual distress syndrome than those who do not suffer from IBS[41-43]. Moreover, IBS patients with dysmenorrhea report noticeably more GI symptoms than non-dysmenorrheic women[41]. In a 10-year follow-up-study conducted in Iceland it has been shown that IBS female patients with dysmenorrhea were twice more likely to have increased symptoms compared to IBS patients without dysmenorrhea[43].

A significant connection between IBS and endometriosis has also been reported[43,44]. Additionally, polycystic ovary syndrome (PCOS), the most common female endocrine disorder affecting up to 10% of reproductive-age women characterized by chronic anovulation and hyperandrogenism, is associated with the increased prevalence of IBS[45-47]. Interestingly, IBS coexisting with PCOS was associated with a higher BMI and percent body fat when compared to PCOS alone[45]. The relationships between obesity, hormonal status and IBS require further investigation, particularly in the context

of obesity being linked with increased inflammatory mediators and in the light of recent reports on the GI dysbiosis[46,48].

### Pregnancy

Pregnancy is characterized by high ovarian hormones levels as well as an increase in opioid-mediated antinociception[3,49]. Little is known, however, regarding IBS symptoms and pregnancy. Many chronic pain syndromes frequently associated with IBS, like migraine headache for example, are alleviated during the time of pregnancy[27]. Similarly, in rodents high ovarian hormones levels during pregnancy reduce somatic and visceral pain sensitivity[50]. During the time of the physiological hyperestrogenemia and hyperprogesteronemia a prolonged GI transit is also observed[51]. Additionally, numerous psychological variables affecting the autonomic nervous system (ANS) may trigger or modulate symptoms reported in pregnant women[52].

### Menopause

Data concerning the impact of the menopause transition on IBS patients remain inconsistent. Although the decline in ovarian hormones may induce or exacerbate GI symptoms, generally, in postmenopausal period, the incidence of IBS decreases significantly[53-55]. However, according to some recent data, IBS symptoms severity may increase after menopause as well[43]. Cain *et al*[26] found that various GI symptoms were reported more frequently by postmenopausal women compared with men, but these differences were not significant when controlled for age. In one study, gas and excessive flatulence were more prevalent in post- than premenopausal healthy women[53].

### Hormone supplementation

Premenopausal healthy women taking oral contraceptives (OCs), monophasic or triphasic preparations, report a typical increase in GI symptoms at menses[55]. However, women with IBS taking OCs, which contain both estrogen and progestin, appeared to have reduced levels of abdominal symptoms compared with IBS women not taking OCs[55]. At the same time, the pattern of GI and non-GI symptoms over the menstrual cycle was similar in female patients with IBS, regardless of OCs use or the predominant bowel pattern[55]. Noteworthy, in women with dysmenorrhea that may coexist with IBS, OCs often reduce the symptoms[15]. Recently, Bird *et al*[56] reported an increased risk for development of IBS with drospirenone. Drospirenone is a synthetic progestin approved in combination with ethinyl-estradiol as an OC. Although it was designed as an antimineralocorticoid steroid, it exhibits antiandrogen activity[56]. In another study evaluating the effect of hormone supplementation on IBS symptoms, the therapeutic efficacy of gonadotropin-releasing hormone agonist (leuprolid) in female patients with menstrual cycle-related symptoms has been reported[57]. However, the use of this medication is limited by its side effects[57].

Based on the recent meta-analysis, there are insufficient data to determine the exact effect of hormone supplementation during menopause on IBS symptoms[19]. In postmenopausal women, hormone replacement therapy (HRT) has been reported to be associated with the increased prevalence of IBS. HRT may prolongs IBS symptoms to a later age or even induce changes in GI function in females not previously affected[58]. One of the confounding factors may be related to the fact that women with IBS are more likely to report various pre- and postmenopausal symptoms, and thus may be prescribed HRT to a greater degree. However, Ruigómez *et al*[58] have shown that both current and past users of HRT presented an increased risk of IBS compared to non-users, even after adjusting for comorbidity and consultation pattern. This increased risk was irrespective of treatment duration, regimen or route of administration of HRT[58].

### Gynecological surgery

There are few data concerning the prevalence of oophorectomy or hysterectomy in IBS female patients, mostly because these surgical procedures are excluding factors in the studies of IBS patients. However, it has been reported that the rate of hysterectomy is about twice higher in women with IBS compared to controls[59]. It is conceivable that IBS patients, because of the chronic abdominal pain, are more likely to be qualified for various surgical procedures (not only gynecological, but also GI surgery like cholecystectomy and appendectomy)[59]. In fact, in a number of women, GI symptoms emerge for the first time after gynecological surgery[60]. Preclinical studies however remain controversial. There is indeed evidence in mice that ovariectomy generates a slow developing and persistent hyperalgesic state localized to the abdomen, lower limbs and abdominal viscera, which is reversed by estrogen supplementation[61,62]. In contrast, in rats, ovariectomy decreased the magnitude of the visceromotor response to colorectal distension compared with cycling rats[63] and abolished restraint stress-induced visceral hypersensitivity[64]. The sensitivity to colorectal distension and the influence of stress on visceral pain were restored by estrogen replacement at a dose comparable to the proestrus level[65].

### Male sex hormones

Most of the explanations of sex-related differences in IBS have focused on the concept that women might be more susceptible, while less attention has been given to the concept that male hormones may be protective against pain disorders including IBS[66]. Androgens, higher in males than females, appear to protect against the development of chronic pain disorders in humans, and testosterone exerts an analgesic effect in experimental pain models, in both men and women[67-69]. Differences in androgen levels, their receptors as well as sites of action may play a role in the sex difference in the risk of developing chronic pain disorders. There are only few reports concerning the role of sex hormones in male

patients with IBS[67,70,71]. Houghton *et al*[66] found that testosterone levels, although similar in the patient and control groups, correlated negatively with perceptual thresholds of rectal distension and overall well-being in IBS patients. In the same study it was found that middle-aged male IBS patients tended to have lower levels of luteinizing hormone compared with male control subjects[66]. Kim *et al*[70] have also reported that the sex hormone status in young male patients is different from that of older male patients and that an elevated sex hormone-binding globulin level might play a key role in the pathophysiology of IBS in young men. Interestingly, a highly significant reduction in male-trait scores in men with IBS has been confirmed[71]. Another unique model to study the relationship between sex hormones and chronic pain was proposed by Aloisi *et al*[72] who evaluated the results of sex-crossed hormone administration in transgender subjects. About half of the female-to-male subjects treated with testosterone reported a significant improvement of the chronic pain (*e.g.*, headache) present before the treatment. Conversely, about one-third of the male-to-female subjects receiving estrogen/anti-androgen treatment developed chronic pain including headaches, breast and musculoskeletal pain, and in some cases visceral pain as well[72]. These findings support experimental and clinical data suggesting that sex steroid hormones play a crucial role in pain perception and modulation.

## SEX HORMONE MODULATION OF THE BRAIN-GUT AXIS AT THE CENTRAL NERVOUS SYSTEM LEVEL

### *Estrogens*

The abundant distribution of estrogen receptors (ERs) at all levels of the brain-gut axis, including the central nervous system (CNS), spinal cord, and the enteric nervous system supports the multiplicity of neuronal action[73]. There are two subtypes of ERs: ER-α and ER-β. Estrogens, similarly to progesterone and testosterone, exert their function by binding to either specific intracellular (nuclear) receptors that act as ligand-dependent transcription factors (classical mechanisms) or membrane-bound receptors (mERs) that stimulate several signal transduction pathways (non-classical mechanisms). The family of nuclear receptors mediate rather slow genomic action of estradiol resulting in enhancement or repression of gene transcription and thus protein synthesis alterations. In contrast, mERs are involved in the rapid action of estrogens related to the activation of various protein-kinase cascades and phosphorylation of proteins, but estrogenic rapid signaling can also occur by recruiting intracellular pathways that can act *via* the genome through phosphorylated cyclic adenosine monophosphate (cAMP) response element protein (pCREB) and intermediate early genes[74]. In addition to the well described G protein-coupled receptor (GPR30), multiple mERs have recently been discovered, such as the classical nuclear ER-α and ER-β, ER-αΔ4, ER-X and mER-Gαq[74-76].

ERs are spread throughout the brain, including the amygdala, hypothalamus, pituitary, hippocampus, cerebral cortex, mid-brain, and brain stem, providing neuroanatomical support for potential numerous target sites of estrogen actions on neurocognitive processes[73,77]. Based on the results of brain imaging studies, greater responsiveness of emotional arousal circuits in relation to visceral pain has been implicated as induceing central mechanisms of pain amplification in IBS, with female subjects showing greater response than male subjects[78]. Recent results confirmed sex differences in emotion-related cognitive processes and functioning of brain networks including the prefrontal regions, cingulate, insula, and amygdala in IBS and healthy control subjects[79].

Estrogens may act in the CNS through multiple pathways modulating production and action of neurotransmitters, influencing electrical excitability and synaptic function, and changing the morphological features of neural elements involved in the function[77,80,81]. Estrogens have been documented to exert differential, sometimes opposite effects on pain. Clinical and experimental data indicate that both analgesic and hyperalgesic responses can be induced by estrogens depending upon the experimental conditions[67]. Estrogens were shown to enhance neuronal system activities during development and in adult life, for instance through the hippocampal neuronal circuits involving acetylcholine, glutamate and brain-derived neurotrophic factor[82]. Noteworthy, elevated levels of estrogens in fertile women have been associated with the increased number of μ-opioid receptors in the brain regions related to pain processing[68]. There is accumulating evidence that estrogens have a significant impact on neuronal plasticity-related process and ameliorate recovery after chronic stress (Table 2)[73].

Estrogens may also contribute to the important sex differences in the stress-related hypothalamic-pituitary-adrenal (HPA) axis response that have been documented in a number of clinical and experimental studies[83]. The menstrual cycle phases, menopausal status and pregnancy have been shown to affect the HPA axis as well as ANS functions[5]. Women between puberty and menopause usually show lower HPA axis and autonomic responses to psychological stressors than men of the same age[84]. However, the HPA axis response to psychological stressors is higher in the luteal phase, when post-stress free cortisol level approaches that for men[84]. CRF is a key mediator of the HPA axis and the brain-gut axis response to stress at both central and peripheral levels[9,85,86]. The co-localization of ER-α with CRF receptors in the hypothalamus represents one of the possible neuroendocrine interactions between CRF signaling pathways and estrogens[87]. Importantly, activation of both receptors ER-α and ER-β has been shown to stimulate *CRF* gene expression in the hypothalamic paraventricular nucleus (PVN)[83,88]. Additionally, estrogens induce also an increase in glucocorticoid receptor expression in the amygdala[88]. In the recent functional magnetic resonance imaging study, it was demonstrated that significant sex differences in brain activity in stress

**Table 2 Sex hormone modulation of the brain-gut-microbiota axis**

| Level of the brain-gut-microbiota axis | Estrogen | Progesterone | Testosterone |
|---|---|---|---|
| Central nervous system | Analgesic or hyperalgesic effect[67]<br>Excitatory action on neurons[72]<br>Estrogen-induced increase in the number of μ-opioid receptors[68]<br>Enhancement of serotonergic postsynaptic responsiveness in the brain[8]<br>Central interaction with CRF signaling pathways-modulation of stress responsiveness[87,89]:<br>Stimulation of *CRF* gene expression in PVN[83]<br>Increase in glucocorticoid receptor expression in the amygdala[83]<br>Influence on neuronal plasticity-related processes[73] | Activation of the γ-aminobutyric acid (GABA) receptors, major inhibitory receptors in the brain[77]<br>Neuroprotective action in the hippocampus[80] | Analgesic effect[72]<br>Inhibition of stress-induced ACTH release[103] |
| Autonomic nervous system | Attenuation of sympathetic responsiveness[108] | Reduced cholinergic responsiveness[5] | Regulation of parasympathetic tone[110] |
| Enteric nervous system/ Gut immune system | Expression of estrogen receptors in enteric neurons, regulation of neurogenic reflexes[73]<br>Activation of colonic NK1 receptors in stress-induced visceral hypersensitivity[64]<br>Augmentation of mast cells secretion[118]<br>Effects on both pro- and anti-inflammatory pathways[113]<br>Peripheral interaction with CRF signaling pathways, modulation of colonic motor and sensory responses to stress[87]<br>Regulation of 5-HT3 receptor expression in rat colon[120]<br>Regulation of secretory and absorptive function of gastrointestinal epithelial cells[128]<br>Enhanced expression of trans-membrane tight junction protein in non-inflamed colon[124]<br>Decreased production of proinflammatory cytokines in experimental colitis in female rats[125,126] | Inhibition of gastrointestinal motility[130]<br>Inhibition of visceral signaling following colonic inflammation[100]<br>Inhibition of mast cells degranulation[131]<br>Immunosuppressive action related to inhibition of NFκB activation in macrophages[133] | Stimulation of smooth muscle contractions[135]<br>Decreased production of proinflammatory mediators inducing visceral hyperalgesia[69,136]<br>No effect on mast cells degranulation[137]<br>Decreased TLR4 expression of macrophages and monocytes[138] |
| Gut microbiota | ER-β expression affects the gut microbiota composition[143]<br>Microbial β-glucuronidase activity determines estrogens deconjugation enabling their reabsorbtion *via* enterohepatic circulation[146]<br>Direct effect on bacterial metabolism, growth and expression of virulence factors[132] | Direct effect on bacterial metabolism, growth, and expression of virulence factors[132] | Reversible 17β reduction of androgens may regulate testosterone level[148]<br>Commensal microbiota-dependent testosterone production protects against autoimmune disease in mice[149] |
| | Bacterial hydroxysteroid dehydrogenase regulates the balance between active and inactive steroids[132] | | |

ACTH: Adrenocorticotropic hormone; CRF: Corticotropin-releasing factor; NFκB: Nuclear factor κB; PVN: Paraventricular nucleus; TLR4: Toll-like receptor 4.

response circuitry were dependent on women's menstrual cycle phase[89]. In addition, chronic treatment with estrogens modulates brain circuitry responsive to stress[90]. Furthermore, administration of estradiol and progesterone directly to the amygdala in rats increases pain response to visceral stimulation suggesting that an amygdala-dependent mechanism may be responsible, at least in part, for the exacerbation of visceral symptomatology in females[91]. A recent meta-analysis by Tillisch *et al*[92] points to the amygdala, a brain region known to facilitate HPA axis output, as one of the most consistently activated areas following rectal stimulation in IBS patient compared with controls. Of significance, the activation of the amygdala by corticosterone eliminates spontaneously occurring differences in visceral and somatic pain perception in cycling female rats, resulting in visceral hypersensitivity during metestrus/diestrus, and increased somatic sensitivity during both metestrus/diestrus as well as proestrus/estrus[39]. This observation could explain the lowered pain thresholds and higher incidence of somatic pain observed in women with IBS[39].

Childhood trauma (early adverse life event, EAL) is associated with changes in HPA axis responsiveness in IBS[93]. Dysregulation of the HPA axis in IBS patients has been related to blunted adrenocorticotropic hormone (ACTH) levels and enhanced cortisol response to visceral stimulation[94]. However, little is known on sex-differences in EAL-induced visceral pain. Interestingly, sexually dimorphic effects of unpredictable EAL on visceral pain behavior in a rodent model has been demonstrated[95]. Female rats exposed neonatally to different pairings of an odor and shock developed visceral hypersensitivity in adulthood, while in contrast, in male rats, visceral sensitivity was not significantly different after EAL. Visceral sensitivity following unpredictable EAL was reversed by ovariectomy and reestablished by estradiol

replacement. These data suggest estrogen-mediated pivotal mechanisms in maintaining visceral hypersensitivity[95].

The serotonergic system represents another potential contribution to sex differences in pain modulation[3,8]. In the CNS, serotonin (5-HT) generally has been associated with descending pain inhibition, whereas in the periphery, 5-HT is an inflammatory mediator and is generally pronociceptive and prokinetic. Estrogens enhance serotonergic postsynaptic responsiveness in the brain[8]. Additionally, estrogens enhance 5-HT synthesis in most part of the brain by increasing expression of the enzyme tryptophan hydroxylase and decreasing the expression of the serotonin re-uptake transporter[96]. The serotonergic and reproductive endocrine systems are also prominently involved in both the regulation of mood and behavioral states. In addition, interactions between these systems have profound implications for the etiology and treatment of anxiety disorders[97]. A growing body of evidence also indicates sex-dependent differences in serotonin-related genetic polymorphisms in IBS patients[98], particularly with regard to anxiety and depressive disorders more common in women with IBS[99].

### Progesterone

The role of progesterone in sex-related differences in pain modulation is less clear. Progesterone activates intracellular receptors to regulate genomic processes, and also affects cell membrane receptors, especially in neurons[100]. Membrane progesterone receptors present in the hippocampus were suggested to contribute the neuroprotective action of the hormone[80]. At the CNS level, progesterone action seems to be dependent on the activation of the γ-aminobutyric acid receptors that are major inhibitory receptors in the brain[101].

### Androgens

While estrogens are commonly indicated as CNS stimulant, androgen receptor-mediated actions are often related to CNS inhibition, which may underlie the lower incidence of many forms of chronic pain in men[72]. Androgens participate in the regulation of the HPA axis response to chronic stress and the autonomic circuitry[102]. Optical and electron microscopic immunocytochemical studies in rodents have revealed that the distribution of androgen receptors is overlapping with that of ER-α, ER-β, as well as progesterone receptors in three major autonomic regions in the brain: the rostral ventrolateral medulla, nucleus of the solitary tract and PVN[80]. In male rats, testosterone inhibits the acute restraint stress-induced ACTH release[103] that, ultimately, may impact on other brain stress-related CRF-mediated influence on colonic motility and visceral pain[9].

## SEX HORMONE EFFECTS ON THE AUTONOMIC NERVOUS SYSTEM

### Estrogens

Estrogens influence also nociceptive pathways at the level of primary afferent nerves and spinal cord projections[104]. A direct involvement of ERs in nociceptive transmission is possible *via* their activation of enkephalin synthesizing cells in the superficial laminae of the spinal cord[105]. Additionally, estrogens modulate the responsiveness of primary vagal afferents neurons to substance P and the activation of glutamate receptors involved in the afferent pain pathways[40]. Spinal estrogen receptors ER-α and ER-β have been also shown to contribute to the facilitation of N-methyl-D-aspartate-dependent colon-to-urethra cross-organ reflex sensitization, which is presumed to underlie pelvic viscero-visceral referred pain[106].

Autonomic dysregulation in response to a visceral stressor is an objective physiologic correlate in IBS[107]. Tillisch *et al*[108] reported gender differences in the ANS reactivity to colorectal distension in IBS patients, with men demonstrating increased sympathetic nervous system activation and decreased parasympathetic activation compared to women. There are also data indicating menstrual cycle-linked differences in the ANS tone that are likely to result from estrogen exposure, and its attenuating influence on sympathetic responsiveness[109]. Furthermore, many other chronic pain syndromes, frequently coexisting with IBS can be also related to autonomic disturbances[28].

### Progesterone and androgens

Progesterone has been shown to reduce cholinergic responsiveness[5]. However, little is known about the effect of testosterone on the ANS. Recently, it has been noticed that testosterone deficiency is accompanied by a decrease in basal parasympathetic tone and reduced baroreflex sensitivity in men with heart failure[110].

## SEX HORMONE ACTIONS AT THE ENTERIC NERVOUS AND GUT IMMUNE SYSTEMS

### Estrogens

Within the enteric neurons of the colon, where both CRF receptor subtype 1 ($CRF_1$) and ERs are expressed, interactions between CRF signaling pathways and estrogens participate in the stimulation of the colonic motor function[90]. Additionally, a local paracrine/autocrine pro-inflammatory action by $CRF_1$ receptor activation was reported in several models of intestinal inflammation both *in vitro* and *in vivo*, as well as the up-regulation of CRF and $CRF_1$ expression in immune cells of the human colonic lamina propria in response to inflammation[86].

There is compelling evidence suggesting an up-regulated gut immune function in patients with IBS, particularly with post-infectious IBS[111]. Gastrointestinal inflammation seems to be strongly modulated by stress, especially in IBS patients being characterized by enhanced stress responsiveness[15]. Important sex-related differences in IBS patients related to neuroimmune inter-

actions have been suggested[71,112]. Female sex is an independent risk factor for developing postinfectious IBS[3]. The following observations support sex differences in immune response: females produce stronger cellular as well as humoral immune reaction, have a greater resistance to bacterial infections, and are more likely to develop autoimmune diseases compared to men, symptoms of which depend on hormonal status[113]. Estrogens may influence both pro- and anti-inflammatory pathways. The effect of estrogens in inflammatory responses has been found extremely complex and dependent on the estrogen level, the cell type, specific inflammatory factors, the type of tissue that is inflamed, the time course of the inflammatory response (*e.g.*, acute *vs* chronic), and the time point at which estrogen exposure occurs[113]. In an experimental model, estrogens contributed also to the colonic neurokin-1 receptor-mediated effects of stress-induced visceral hypersensitivity to colorectal distension[64].

Mast cells represent another crucial link in sex-dependent neuroimmune interactions as they co-express CRF and sex hormone receptors[114-116]. The number of colonic mucosal mast cells was found to be higher in female compared to male IBS patients[10]. Mediators released by activated mast cells, characterized by extensive anatomical and functional communication with intrinsic and extrinsic nervous system of the gut, evoke visceral hypersensitivity and increase mucosal permeability[117]. Notably, mast cells are involved in many other disorders, frequently overlapping with IBS such as fibromyalgia, interstitial cystitis, chronic fatigue syndrome and migraine, all of which occur more often in women, are exacerbated during ovulation and reduced during pregnancy[10]. These sex-related differences in the prevalence and severity of chronic pain disorders could be related to the fact that mast cells express progesterone and estrogen receptors[10]. Estradiol has been shown to augment mast cells secretion, whereas tamoxifen (an estradiol receptor antagonist) inhibits this function[118].

The serotonergic system at the peripheral level may also contribute to sex differences in modulation of GI motility, secretion and sensitivity[3,8]. Fluctuations in estrogen levels during ovarian cycle cause predictable changes in 5-HT system in women[8]. Moreover, 5-HT concentration varies with sex and menstrual status in patients with diarrhea-predominant IBS[119]. Experimental studies indicate that colonic 5-$HT_3$ receptor gene expression is increased in ovariectomized rats exposed to restraint stress and restored with hormone replacement after ovariectomy[120]. Recently, Galligan *et al*[121] proposed serotonine transporter (*SERT*) gene knockout (KO) rats as a new interesting model for studying interactions between serotonin, sex, and visceral sensation. SERT KO female rats display an increased colonic extracellular serotonin associated with visceral hypersensitivity and hyperexcitability of colon projecting sensory neurons, which is not observed in male SERT KO rats[121]. Gender difference has been also shown in SERT activity and serotonin concentration in platelets of IBS patients[122].

Estrogen-dependent modulation of the intestinal barrier function is another component in sex-related differences in IBS. It has been well established that stress involving the activation of $CRF_1$ receptors alters intestinal barrier that appears to be a prerequisite for the development of visceral hypersensitivity in both human and rodents[6,123]. In the colon, ERs signaling enhances expression of trans-membrane thigh junction proteins in non-inflamed conditions[124], and decreases production of proinflammatory cytokines in experimental colitis[125,126]. In human, acute experimental stress evokes a differential gender-dependent increase in intestinal macromolecular permeability[127]. A significant increase in albumin permeability in healthy women, but not in men, could explain enhanced female susceptibility to IBS[127].

Additionally, ERs are localized on the epithelial cells throughout the GI mucosa and may affect secretory and absorptive functions[37,128,129]. The fluid retention that occurs in females during the cycle may be associated with the extra-nuclear action of estrogen that can stimulate calcium entry into colonic epithelial cells as well as suppress c-AMP-dependent chloride secretion in the distal colonic epithelium in females only, both in rats and humans[128].

### Progesterone and androgens

At the peripheral level progesterone has been suggested to influence both visceral sensitivity and motility *via* prostaglandins[100]. Overexpression of progesterone receptors in colonic muscle in women with slow transit constipation is associated with lower levels of prostaglandin $PGF_{2\alpha}$ and tromboxane A that cause muscle contraction, and higher levels of PGs that cause muscle relaxation (such as $PGE_2$)[130]. Progesterone may also inhibit estrogen-dependent mast cells degranulation[131]. Progesterone receptors have been identified in epithelial cells, granulocytes, macrophages, and lymphocytes[132]. Progesterone is known to exert an immunosuppressive action as it inhibits the activation of nuclear factor (NF)κB and increases the expression of the suppressor of cytokine signaling protein (COS1) in macrophages[133].

Testosterone and its active metabolite 5α-dihydrotestosterone are potent modulators of colonic motility by stimulating smooth muscle contractions through non-genomic calcium sensitization pathways[134]. In the urethral calculosis model of visceral pain, Aloisi *et al*[135] did not find any significant effect of testosterone on visceral pain. However, there is growing body of evidence that androgens may contribute to the modulation of visceral pain by decreasing pro-inflammatory mediators that participate in the development of hyperalgesia[69,136]. Apart from female sex hormone receptor expression, mast cells also express androgen receptor, however, testosterone treatment had no effect on mast cell degranulation[137]. Testosterone decreases also the expression of macrophage and monocyte Toll-like receptor 4, which is involved in the activation of the innate system response to pathogen challenge[138].

## INTERACTIONS BETWEEN SEX HORMONES AND THE GUT MICROBIOTA

The increasing knowledge of the role of microbiota in health and disease state has shed a light on the critical role of the enteric microbiota, both commensal and pathogenic organisms, in regulation the brain-gut axis. This has consequently led to the coining of a new term: the brain-gut-enteric microbiota axis[11]. The bi-directional communication between the gut bacteria and the brain occurs through neural, immune, and endocrine pathways[12,48,139,140] which may be modulated by sex hormones, in particular estrogens. In fact, it has been recently reported that the microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner[141]. Clarke *et al*[141] found that male germ free mice, unlike females, display a significant elevation in the hippocampal concentration of 5-HT and its metabolite, compared with conventionally colonized control animals.

Numerous studies have reported the effects of sex hormones on the dimorphic sex differences in the response to microbial and viral infections[132]. Besides the role of sex hormones in the modulation of the immune system, they have a direct effect over bacterial metabolism, growth, and expression of virulence factors. For instance during pregnancy, the proportion of certain bacteria species associated with plaque microbiota is altered with a noticeable increase in the ratio of anaerobic to facultative bacteria[142]. Of significance, recent studies indicate that steroid nuclear receptor expression including ER-β can determine the intestinal microbiota composition[143].

Moreover, the gut microbiota may also affect estrogens metabolisms and their systemic level[144]. Conjugated estrogens are excreted in the bile and pass into the distal ileum, where they are variably deconjugated and may be reabsorbed from the gut lumen and enter the circulation *via* the portal vein[145]. It has been shown in men and postmenopausal women that the intestinal microbiota richness and function, associated for example with β-glucuronidase activity, influence levels of non-ovarian estrogens *via* enterohepatic circulation[146]. Bacteria are capable of metabolizing sex hormones through the activity of various enzymes such as hydroxysteroid dehydrogenase that regulate the balance between active and inactive steroids[130]. In particular, fecal bacteria can perform hydrolytic, reductive and oxidative reactions of estrogens and androgens[147]. Reversible 17β reduction of androgens carried out by the gut microbiota is suggested to play a role in the regulation of testosterone level[148]. The results of a landmark study published recently by Markle *et al*[149] provide astonishing conclusions indicating that sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. In the study performed in the non-obese diabetic mouse model of type 1 diabetes, they showed that male puberty in mice leads to changes in the gut microbiota that reinforce testosterone production, which is protective against the development of T and B cell functions linked to autoimmune disease[149]. In mice, the properties of the male-associated microbiota can be transferred to younger females and exert testosterone-mediated protection from autoimmune disease upon recipients. The observations that early-life microbial exposures determine sex hormone levels and modify sex-mediated immune regulation may have crucial implications for the pathophysiology of IBS. A new concept of "microgenderome" is emerging based on the recent observations that the gender bias present in numerous diseases is not entirely a host-intrinsic factor, but may be exercised and/or reinforced by the commensal microbiota of the host[150]. Undoubtedly, further studies are needed to elucidate the role of microgenderome in IBS.

## THERAPEUTIC IMPLICATIONS

The modulator role of sex hormones on the bi-directional interactions within the brain-gut-microbiota axis may have significant therapeutic implications in IBS. However, although gender differences in responses to treatment modalities exist, the approach to IBS patients in both genders is quite similar so far. Clinical observations confirm that alosetron, a 5-$HT_3$ receptor antagonist, is more effective in improving urgency and loose stools in IBS-diarrhea predominant women than men[120,151,152]. The basis for this noticeable sex difference in therapeutic efficacy of alosetron could be associated with sex-related differences in 5-$HT_3$ receptor expression, lower alosetron clearance in women, and/or greater 5-HT synthesis in certain brain regions in IBS male patients compared with female IBS patients[153]. Sex difference in genetic polymorphism of the 5-HT transporter (SERT) promoter region has been also suggested and may induce the different expression of affective symptoms in women compared with men[154]. Additionally, the potential role of interaction between gonadal hormones and the cytochrome P450 pathway may be considered in sex-related differences in drug clearance[155]. Differences in adipose tissue compartment in women compared to men may affect this process as well[40].

Women with IBS are more susceptible to anxiety and depression and other stress-related disorders. However, in the study comparing the efficacy of treatment with paroxetine alone or combined with psychotherapy, no gender effect was reported[156]. Preliminary observations suggesting that IBS female patients may better respond to hypnotherapy[157] is yet to be confirmed. The recent results of randomized controlled trial have shown that gender, age, disease duration and IBS type have no influence on the long-term success of gut-directed hypnotherapy[158].

Regarding the role of sex hormones in the pathogenesis of IBS, therapeutic approaches aiming to suppress ovarian steroidogenesis have been also considered. In fact, gonadotropin-releasing hormone agonist (leuprolide)

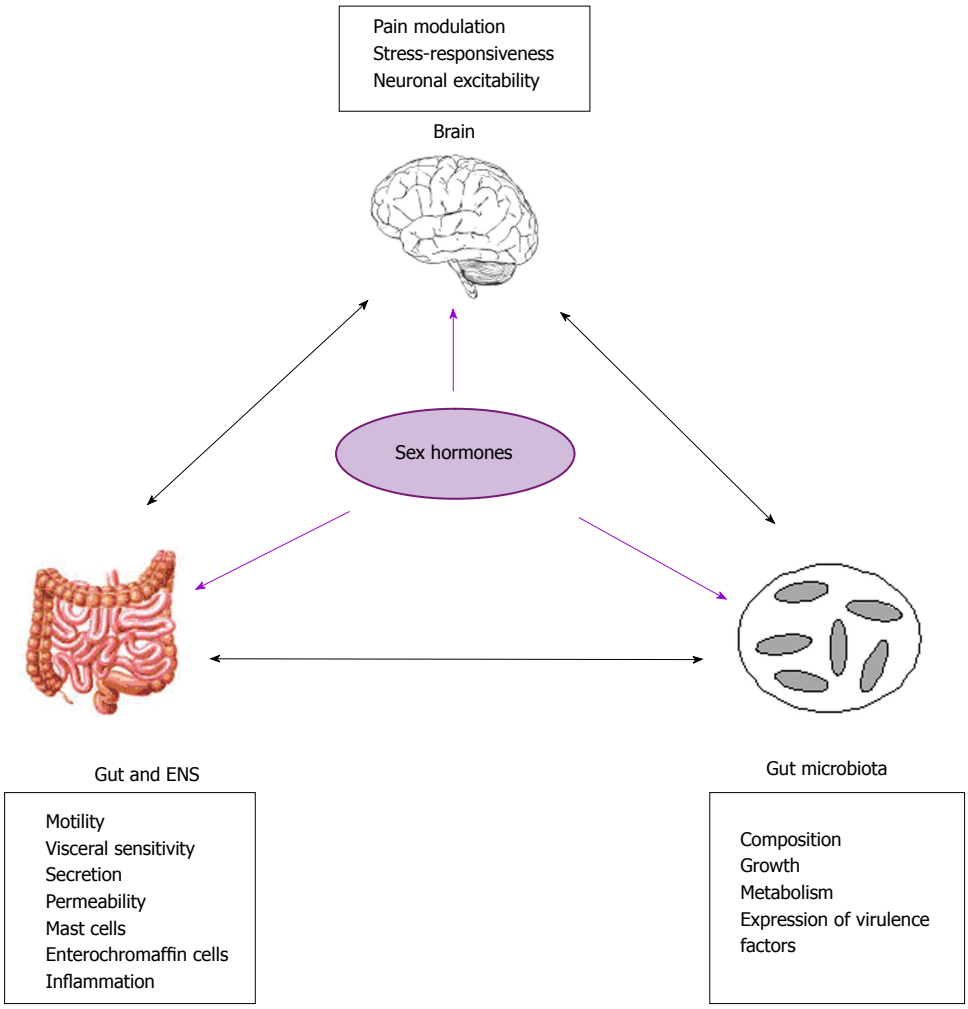


**Figure 1 Sex hormones in the mutual brain-gut-microbiota interactions.** Sex hormones influence peripheral and central regulatory mechanisms involved in the pathophysiology of irritable bowel syndrome contributing to the alterations in stress response, visceral sensitivity and motility, intestinal barrier function, and immune activation of intestinal mucosa. Sex hormones have also a direct effect on the gut microbiota. ENS: Enteric nervous system.

was reported to be effective in IBS female patients with menstrual cycle-related symptoms[57]. Nevertheless, many unpleasant side effects of leuprolide similar to climacteric-like syndrome significantly limit its application[57].

Noteworthy, interactions between gonadal hormones and pain modulation are bi-directional, as pain therapies in different experimental and clinical conditions have been found to affect the gonads as well[159,160]. For example, morphine treatment increased estrogen receptor, androgen receptor and *TRPV1* genes expression in the ovary, whereas in the testis the opiate reduced ER-α and ER-β mRNA expression not affecting androgen receptor and *TRPV1* expression[160].

A pivotal interdependence between the composition and stability of the gut microbiota and GI function as well as stress-related behavioral changes indicate a great therapeutic potential of probiotics, prebiotics and antibiotics in IBS[161,162]. So far, no gender specificity in probiotics efficacy in IBS patients has been reported[163]. Nevertheless, in the light of the microgenderome concept and sex-dependent differences in the immune regulation driven by gut microbiome[150], gender specificity in microflora manipulation seem to be essential and is expected to be extensively explored in the near future.

## CONCLUSION

The results of epidemiological studies and clinical observations confirm significant sex and gender differences in the IBS prevalence and symptomatology. Furthermore, a growing number of clinical and experimental data strongly support a crucial role of sex hormones in the regulatory mechanisms of the brain-gut-microbiota axis involved in the pathophysiology of IBS (Figure 1). Some discrepancies in the results, especially related to the influence of estrogens, may result from different experimental conditions or heterogeneous groups of patients (*e.g.*, different age, menstrual status), but they also reflect the very complex nature of sex hormone actions. Estrogens can induce dual effects, both analgesic or hyperalgesic, as well as pro- or anti-inflammatory. Noteworthy, alterations in estrogen-induced visceral sensitivity seem to depend not only on the gonadal hormones levels, but more so on sudden changes in their levels, their sus-

tained genomic effects, and complex interactions with other neurotransmitters. Concomitant alterations in the number (up- or down-regulation) and sensitivity of ERs may play a crucial role in these processes as well. Thus, the physiological fluctuation in sex hormones may evoke for example different responses in IBS female patients compared to healthy women. Furthermore, a growing body of evidence indicates a protective role of androgens in pain modulation and anti-inflammatory properties of testosterone that may inhibit the development of visceral hyperalgesia. That could contribute to the higher susceptibility of women to IBS. A better understanding of the role of sex hormones in the modulation of the brain-gut-microbiota axis should enable a more effective and sex-tailored therapeutic approach in IBS.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

# Overgrowth of the indigenous gut microbiome and irritable bowel syndrome

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## Abstract

Culture-independent molecular techniques have demonstrated that the majority of the gut microbiota is uncultivable. Application of these molecular techniques to more accurately identify the indigenous gut microbiome has moved with great pace over recent years, leading to a substantial increase in understanding of gut microbial communities in both health and a number of disorders, including irritable bowel syndrome (IBS). Use of culture-independent molecular techniques already employed to characterise faecal and, to a lesser extent, colonic mucosal microbial populations in IBS, without reliance on insensitive, traditional microbiological culture techniques, has the potential to more accurately determine microbial composition in the small intestine of patients with this disorder, at least that occurring proximally and within reach of sampling. Current data concerning culture-based and culture-independent analyses of the small intestinal microbiome in IBS are considered here.



**Key words:** Gut microbiome; Small intestinal bacterial overgrowth; Irritable bowel syndrome

**Core tip:** The majority of the gut microbiota is uncultivable. Use of culture-independent molecular methods, without reliance on traditional microbiological culture techniques, has the potential to determine microbial composition in the small intestine of patients with irritable bowel syndrome. Current data concerning culture-based and culture-independent analyses of the small intestinal microbiome in patients with irritable bowel syndrome are considered here.

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## INTRODUCTION

Culture-independent molecular techniques have demonstrated that the majority of the gut microbiota is uncultivable[1,2]. Application of these molecular techniques to more accurately identify the indigenous gut microbiome has moved with great pace over recent years, leading to a substantial increase in understanding of gut microbial communities in both health and a number of disorders, including irritable bowel syndrome (IBS). Most studies of the gut microbiome in this highly prevalent disorder, characterised by abdominal pain, abdominal distension and altered bowel habit, have to date focussed on analyses of faecal samples and have demonstrated disturbances in a range of bacterial populations in both adults and children with IBS[2-10]. In adults, disturbances in

faecal *Clostridium cocleatum*, *Clostridium coccoides*, *Clostridium thermosuccinogenes*, *Collinsella aerofaciens*, *Coprococcus eutactus*, *Staphylococcus aureus* (*S. aureus*), *Bifidobacterium catenulatum* (*B. catenulatum*), *Ruminococcus torques*, *Ruminococcus bromii*-like, bifidobacteria, Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, *Lactobacillus* spp and *Veillonella* spp have been demonstrated in IBS[2,3,5-10]. In children, a faecal microbiome characterised by a significantly increased percentage of Gammaproteobacteria, including *Haemophilus parainfluenzae*, a novel *Ruminococcus*-like microbe and an increased number of several bacterial taxa from the genus *Alistipes* has been reported in the IBS setting[4]. Analyses of colonic mucosa-associated microbial populations, as determined from mucosal biopsies, suggest compositional differences compared to faecal microbiota may occur in IBS[11] and it has been hypothesised that disturbances at this mucosa-associated level may be more important than those occurring luminally in the pathogenesis of IBS symptoms[12]. In further support of the notion that the gut microbiome participates in the pathogenesis of IBS are the findings of systematic reviews and a meta-analysis, which suggest that probiotics may be of therapeutic value, although results of individual studies are inconsistent and trial designs variable, such that it remains uncertain as to which bacterial species or strains may be of most benefit for which particular symptom component of the IBS complex[13-15].

As opposed to faecal and colonic mucosa-based analyses, possible disturbances in the microbial ecology of the small intestine in patients with IBS have been less well studied. In particular, the prevalence of small intestinal bacterial overgrowth (SIBO) has long remained a matter of conjecture, with concern over the accuracy of diagnostic tests for SIBO one factor clouding this issue. Notably, reported prevalence rates of SIBO in patients with IBS are lower when the diagnosis of SIBO has been based on culture of proximal small intestinal luminal secretions compared to when based on indirect breath hydrogen tests, performed following the ingestion of a fermentable substrate such as lactulose[16]. False-negative culture results have been hypothesised as an explanation for this discrepancy, as a result of SIBO possibly occurring distal to the region of sampling[17]. Conversely, a high false-positive rate of the lactulose breath hydrogen test (LBHT) for SIBO is recognised, based on an initial study performed to investigate the diagnostic accuracy of the LBHT in patients with predisposition to SIBO in which breath testing was combined with scintigraphy[18], recently replicated in the IBS setting[19], that demonstrated that a "positive" result for SIBO may, in fact, result from the test substrate being metabolised by colonic, rather than small intestinal, microbial flora. Sensitivity for culture-proven SIBO has also been shown to be lacking, even with combined scintigraphic assessment[18], such that the LBHT has fallen out of favour as a diagnostic test for SIBO, including in patients with IBS[20].

Another possibility is that disturbances of the small intestinal microbial ecology - either overgrowth or reduced levels of various bacterial species - may, indeed, be present in the region of sampling in patients with IBS but simply not be represented by standard bacteriological culture results, due to the inherent inability to properly demonstrate the gut microbiota in this way. Use of culture-independent molecular techniques already employed to characterise faecal and, to a lesser extent, colonic mucosa-associated microbial populations in IBS, without reliance on insensitive, traditional microbiological culture techniques, has the potential to more accurately determine microbial composition in the small intestine of patients with this disorder, at least that occurring proximally and within reach of sampling. Current data concerning culture-based and culture-independent analyses of the small intestinal microbiome in IBS are considered here.

## PROXIMAL SMALL INTESTINAL MICROBIOTA IN IBS

A total of six published studies have investigated the proximal small intestinal microbiota in well-categorised cohorts of IBS patients and reported findings in relation to IBS-status[16-21]. Four of these studies analysed microbiota in luminal secretions, using standard culture techniques, with one also employing culture-independent molecular methods[21-24]. An additional two studies analysed mucosa-associated microbiota, with both of these using culture-independent molecular techniques[25,26]. Whether there exist compositional differences between small intestinal luminal and mucosa-associated microbial populations in IBS is currently unknown, as no study performed to date has contemporaneously analysed luminal and mucosa-associated microbiota in the same cohort of IBS patients.

### *Assessments of luminal secretions*

Posserud *et al*[21] prospectively investigated 162 consecutive patients in Sweden with a clinical diagnosis of IBS based on Rome II criteria, including 49 (30%) with diarrhoea-predominant IBS (IBS-D), 37 (23%) with constipation-predominant IBS (IBS-C) and 76 (47%) with alternating-type IBS (IBS-A), with culture of a jejunal aspirate obtained *via* the central lumen of a water-perfused manometry catheter after a meal. The mean age of IBS patients was 38 years. Twenty-six healthy subjects (mean age 40 years) served as controls. No subject had been treated with antibiotics within 2 wk prior to the study or had received medications that might affect the gastrointestinal tract within 48 h of assessment. SIBO, defined by viable counts of colonic-type bacteria $\geqslant 10^5$ colony forming units/mL (CFU/mL), was found in 7 patients (4%) (mean age 49 years), including 2/49 (4%) with IBS-D, 3/37 (8%) with IBD-C and 2/76 (3%) with IBS-A. Bacterial isolates in IBS subjects with SIBO variously included *Escherichia coli*, *Enterococcus* species, *Clostridium* species, *Enterobacter* species, *S. aureus* and *Klebsiella* species. The prevalence of SIBO in patients with IBS was comparable to that in asymptomatic controls (1/26;

**Table 1 Studies investigating the prevalence of small intestinal bacterial overgrowth in patients with irritable bowel syndrome, using culture-based assessments of proximal small intestinal luminal secretions**

| Country | IBS patients | Controls | Mean age (yr) | Aspirate details | Definition and prevalence of SIBO in patients and controls | | |
|---|---|---|---|---|---|---|---|
| Sweden[21] | $n$ = 162<br>IBS-D, $n$ = 49<br>IBS-C, $n$ = 37<br>IBS-A, $n$ = 76 | $n$ = 26, healthy | IBS: 38<br>Controls: 40 | Non-fasting; *via* water-perfused manometry catheter from jejunum | ≥ $10^5$ CFU/mL colonic-type bacteria: | IBS patients<br>IBS-D<br>IBS-C<br>IBS-A<br>Controls | 7/162 (4%)<br>2/49 (4%)<br>3/37 (8%)<br>2/76 (3%)<br>1/26 (4%) |
| | | | | | ≥ $5 \times 10^3$ CFU/mL colonic-type bacteria: | IBS patients<br>Controls | 17/162 (11%)<br>1/26 (4%) |
| | | | | | ≥ $5 \times 10^3$ CFU/mL any bacteria: | IBS patients<br>Controls | 70/162 (43%)[1]<br>3/26 (12%) |
| The Netherlands[22] | $n$ = 8 (out of a cohort of 12 symptomatic patients) | $n$ = 9, healthy | Symptomatic: 39<br>Control: 26 | Fasting; *via* weighted catheter from jejunum | > $10^5$ CFU/mL colonic-type bacteria: | Symptomatic patients<br>Controls | 1/12 (8%)<br>0/9 (0%) |
| | | | | | Colonic-type bacteria: *Enterobacteriaceae* ≥ $10^3$ CFU/mL, *Bacteroides* species ≥ $10^2$ CFU/mL or *Clostridium* species ≥ $10^2$ CFU/mL | Symptomatic patients<br>Controls | 1/12 (8%)<br>0/9 (0%) |
| United States[23] | $n$ = 148 | $n$ = 527, symptomatic | Overall: 53 | Fasting; *via* endoscopy from duodenum | ≥ $10^5$ CFU/mL colonic-type aerobic bacteria OR ≥ $10^4$ CFU/mL anaerobic bacteria: | IBS patients:<br>Controls | 2%<br>10% |
| Greece[24] | $n$ = 112<br>IBD-D, $n$ = 35<br>IBD-C, $n$ = 19<br>IBD-A, $n$ = 58 | $n$ = 208, symptomatic | SIBO: 63.6<br>No SIBO: 69.5 | Fasting; *via* endoscopy from duodenum | > $10^5$ CFU/mL colonic-type aerobic bacteria: | IBS patients<br>Controls | 24/112 (21%)[2]<br>11/208 (5%) |
| | | | | | > $10^3$ CFU/mL colonic-type aerobic bacteria: | IBS patients<br>IBS-D<br>IBS-C<br>IBS-A<br>Controls | 42/112 (38%)[2]<br>21/35 (60%)[2,5]<br>6/19 (32%)[3]<br>15/58 (26%)[4]<br>20/208 (10%) |

[1]$P$ = 0.002 compared to controls; [2]$P$ < 0.0005 compared to controls; [3]$P$ = 0.012 compared to controls; [4]$P$ = 0.003 compared to controls; [5]$P$ = 0.001 compared to controls. IBS-D: Diarrhoea predominant-type irritable bowel syndrome; IBS-C: Constipation predominant-type irritable bowel syndrome; IBS-A: Alternating-type irritable bowel syndrome; CFU: Colony forming units; SIBO: Small intestinal bacterial overgrowth; IBS: Irritable bowel syndrome.

4%). Neither did prevalences of SIBO differ significantly between IBS patients and controls when alternative definitions of SIBO were employed (viable counts of any bacteria ≥ $10^5$ CFU/mL, 6% and 4%, respectively; viable counts of colonic-type bacteria ≥ $5 \times 10^3$ CFU/mL, 11% and 4%, respectively). Conversely, viable counts of any bacteria ≥ $5 \times 10^3$ CFU/mL were significantly more common in the IBS cohort than in healthy controls (43% *vs* 12%). While water perfusion through the manometry catheter may have diluted the absolute values of viable bacterial counts obtained, and the ingestion of a test meal prior to sampling for bacteriological analysis may, alternatively, have increased these values compared to those that may have been recovered under fasting conditions, any differences between IBS patients and controls were unlikely explained on these bases, as subjects were studied under identical conditions (Table 1).

As expected since small intestinal dysmotility typically promotes SIBO with colonic-type bacteria[27], this increased prevalence of mildly elevated non-colonic-type bacterial counts in IBS patients reported by Posserud *et al*[21] could not reliably be accounted for by small intestinal dysmotility, as assessed by manometry. Notably, the use of proton pump inhibitors (PPIs) and other drugs that reduce gastric acidity was not controlled for prior to 48 h of study and, given that IBS patients are often treated with such drugs, it is possible that the mildly elevated, non-colonic-type viable bacterial counts found in the IBS cohort may have occurred as a consequence of treatment of symptoms rather than as an initial cause of symptoms. Such a possibility could not be assessed by this study design.

Kerckhoffs *et al*[22] in The Netherlands subsequently reported on 12 symptomatic patients, including 8 with IBS, and 9 healthy subjects, from whom a fasting jejunal aspirate could be obtained using a weighted catheter after infusion of 10 mL of normal saline. Studied IBS patients within the symptomatic group and controls came from initial cohorts of 10 IBS patients (mean age 39 years) and 11 controls (mean age 26 years), respectively, with two IBS patients and two controls ultimately excluded as a jejunal aspirate could not be obtained. Aspirates were subjected to both standard culture and molecular-based analyses, the latter following deoxynucleic acid (DNA) extraction and quantitative polymerase chain reaction (PCR) amplification. No antibiotics were permitted in the two weeks prior to study, although PPI's were permitted until the day before study. With regard to culture results and notwithstanding the possibility of dilution by the saline infusion, SIBO, as defined by a viable colonic-type bacterial count > $10^5$ CFU/mL, was present in 1/12 (8%) of the symptomatic group (the 8 IBS patients within the

symptomatic group were not separately reported) and none of the 9 controls. Using an alternative definition still based on colonic-type bacteria (*Enterobacteriaceae* $\geqslant 10^3$ CFU/mL or *Bacteroides* species $\geqslant 10^2$ CFU/mL or *Clostridium* species $\geqslant 10^2$ CFU/mL), the prevalence of SIBO remained 1/12 (8%) in symptomatic patients and 0/9 (0%) in controls. Moreover, no significant difference in median total viable bacterial counts between symptomatic patients and healthy controls was apparent. Similarly, no significant difference in the total bacterial DNA count between symptomatic patients and healthy controls was evident, while PCR analysis demonstrated that levels of the colonic-type flora, *Enterobacteriaceae*, *Faecalibacterium prausnitzii*, *Bacteroides fragilis* and *Clostridium coccoides*, were also comparable in the symptomatic and healthy groups. Sub-analyses in relation to IBS-D, IBD-C and IBD-A status were not included.

In another analysis, Choung *et al*[23] undertook a retrospective assessment of 675 symptomatic patients in the United States who had undergone culture of a duodenal aspirate, obtained endoscopically under fasting conditions, to assess for possible SIBO, including 148 (22%) patients with a clinical diagnosis of IBS. By comparison to the studies from Sweden and The Netherlands[13,14], the mean age of study subjects in this analysis was older (53 years) and no asymptomatic controls were included. The IBS patients included did not represent a consecutive cohort, but rather a select group attending an academic institution whose physicians deemed symptoms troublesome enough to warrant microbiological assessment. SIBO, defined by a viable colonic-type aerobic bacterial count $\geqslant 10^5$ CFU/mL or an anaerobic viable count $\geqslant 10^4$ CFU/mL, was present in only 2% of the IBS group. The species of the overgrowth bacteria isolated from patients with SIBO were not reported. Placed in context, a diagnosis of IBS was associated with an odds ratio for an abnormal aspirate result in keeping of SIBO of only 0.2 (95%CI: 0.1-0.7) compared to the likelihood of SIBO in patients with non-IBS diagnoses, including inflammatory bowel disease, pancreatitis and small intestinal diverticula, which were associated with three-fold, nearly five-fold and over seven-fold increases in odds for SIBO, respectively. Overall, the likelihood of SIBO was significantly related to older age, with the mean age of those with SIBO found to be 66 years. A substantial number of studied IBS patients were taking a PPI at the time of assessment and the proportion of this subgroup that was found to have SIBO remained low (2%). Conversely, the proportion of IBS patients with detectable viable bacterial counts in duodenal secretions, although not sufficient to fulfil criteria for SIBO, was five-fold higher in the setting of PPI use (15%) than in the absence of PPI use (3%), in keeping with the concept that proximal small intestinal microbial ecology may be disturbed by such therapy, even if not to a degree to constitute SIBO as commonly defined. Data in relation to IBS-D, IBD-C and IBD-A sub-categories of IBS were not provided.

A fourth culture-based study investigated the prevalence of SIBO in a consecutive cohort of 320 symptomatic patients undergoing outpatient upper gastrointestinal endoscopy in Greece, including 112 (35%) with a diagnosis of IBS according to Rome Ⅱ criteria (IBS-D: $n$ = 35, 31.2%; IBD-C: $n$ = 19, 16.9%; IBD-A: $n$ = 58, 51.8%)[24]. Most common indications for endoscopy in IBS patients were dyspepsia ($n$ = 75, 66.9%), anaemia ($n$ = 24, 21.4%) and change in bowel habit ($n$ = 9, 8.0%). Aspirates for microbiological assessment were obtained endoscopically under fasting conditions from the third part of duodenum. The prevalence of SIBO, defined by $> 10^3$ CFU/mL of colonic-type aerobic bacteria, was significantly higher in IBS patients than non-IBS patients (42/112, 38% *vs* 20/208, 10%). Among the IBS cohort, SIBO was present in 21/35 (60%) with IBS-D, 6/19 (32%) with IBS-C and 15/58 (26%) with IBS-A. *Escherichia coli* was the colonic-type bacterial species most commonly isolated in IBS patients with SIBO. Using a more restrictive definition of SIBO of $> 10^5$ CFU/mL of colonic-type aerobic bacteria, the prevalence of SIBO remained significantly higher in patients with IBS (24/112, 21%) than in those without IBS symptoms (11/208, 5%). The mean age of patients enrolled in this study was higher than that in the other three above-mentioned reports, with values of 63.6 years and 69.5 years in the SIBO and non-SIBO groups, respectively. That the highest prevalence of SIBO in IBS patients in the four studies discussed here should be found in the oldest of the four study cohorts, especially in those older patients with IBS-D, is in keeping with a previous report demonstrating a high prevalence of SIBO with colonic-type bacteria, including *Escherichia coli*, in the symptomatic elderly, including those with otherwise unexplained chronic diarrhoea[28]. Notably, distinct age-related disturbances in faecal microbiota, including increased levels of *Escherichia coli*, have also recently been demonstrated in the elderly[29].

### Assessments of mucosa-associated microbiota

Kerckhoffs *et al*[25] investigated 41 patients with IBS fulfilling Rome Ⅱ criteria, including 14 (34%) with IBS-D, 11 (27%) with IBS-C and 16 (39%) with IBS-A, and 26 healthy controls. The mean age of IBS subjects was significantly older than that of controls (42 years and 31 years, respectively). Duodenal brushings were obtained and samples were subjected to DNA extraction and PCR amplification. Based on detection of significantly lower levels of *B. catenulatum* in faecal samples of the IBS cohort, the authors focussed on whether contemporaneous duodenal mucosal levels of *Bifidobacterium* species were similarly disturbed. A significant reduction in duodenal mucosa-associated *B. catenulatum* levels as a percentage of total duodenal mucosa-associated bifidobacterial loads was found in the IBS group (4.85% ± 0.5%) compared to healthy controls (17.04% ± 2.3%), with this relationship consistent across all three IBS subgroups. By contrast, levels of *B. adolescentis*, *B. bifidum* and *B. longum* did not dif-

fer significantly between healthy subjects, IBS patients or IBS subgroups.

In a subsequent case-control analysis, the same authors collected duodenal mucosal brush and faecal samples from 37 IBS patients (mean age 42 ± 2.3 years), including 13 (35%) IBS-D, 11 (29%) IBS-C and 13 IBS-A (35%), and 20 healthy controls (mean age 32 ± 2.6 years)[26]. Bacterial 16S rRNA gene was amplified and analysed using PCR denaturing gradient gel electrophoresis (DGGE). Pooled average DGGE profiles were generated and fingerprints compared. DGGE band fragments confined to healthy or IBS patient groups were further characterised by sequence analysis. Significantly higher levels of *Pseudomonas aeruginosa* were evident in duodenal brushings of the IBS patients than in healthy subjects (8.3% ± 0.95% of clones *vs* 0.1% ± 0.069% of clones, respectively), a trend replicated in paired faecal samples and across all IBS-subtypes. While antibiotic pre-treatment has been shown to increase the colonisation potential of *Pseudomonas* species[30], it is notable that no antibiotic therapy was permitted within one month of study in this analysis. Nonetheless, it remains to be determined whether the elevated levels of *Pseudomonas aeruginosa* reported by the authors are of pathophysiological relevance or merely epiphenomenal, perhaps related to the reduced expression of *B. catenulatum* previously reported or other factors yet to be defined.

### *Effect of antibiotic therapy on small intestinal microbiota and symptoms in IBS*

Randomised trials of the orally administered antibiotics, neomycin and rifaximin, have separately demonstrated a reduction in IBS symptoms in non-IBS-C patients following antibiotic treatment[31-34]. Nonetheless, whether or not treated patients had SIBO and whether antibiotic use was associated with a reduction in viable small intestinal bacterial counts or microbial compositional change that correlated with symptom improvement was not assessed.

To date, only one study has investigated the impact on antibiotic therapy on SIBO and symptom improvement in patients with IBS[21]. In that analysis, seven patients with culture proven SIBO in jejunal secretions (mean age 49 years) were treated with oral ciprofloxacin, 500 mg twice daily for 10 d. Follow-up cultures following antibiotic treatment showed decreased viable bacterial counts in five patients (71%), although four (57%) still fulfilled criteria for SIBO. Three patients (43%) reported at least a 25% improvement in IBS symptoms following the course of ciprofloxacin, but IBS symptom responder status was not consistently related to reduction in small intestinal luminal viable bacterial counts. Whether symptom responder status may have correlated more closely with any antibiotic-related changes in faecal or colonic microbiota was not assessed.

No data are currently available with regard to the possible impact of antibiotic therapy on duodenal mucosa-associated composition and whether any antibiotic-related compositional change in the duodenal mucosa-associated microbial community correlates with symptom improvement in patients with IBS.

### *Efficacy of probiotic regimens that include microbiota shown by molecular techniques to be deficient in IBS*

The health benefits of *B. catenulatum* for the host, if any, are currently unknown. However, members of the bifidobacteria group are often included in probiotic regimens used for the treatment of IBS[35]. Trials of probiotics that specifically include *B. catenulatum* and any other small intestinal mucosa-associated bacterial species that may be shown in future to be reduced in patients with IBS will be of considerable interest, from both therapeutic and disease mechanism perspectives.

## CONCLUSION

Current microbial data, although relatively limited and based predominantly on culture-based assessments of luminal secretions, suggest that only a minority of patients with IBS have luminal SIBO, irrespective of the definition employed, with the exception of older subjects with the diarrhoea-predominant form. Available data obtained from a relatively young cohort demonstrating that symptom improvement following antibiotic therapy in IBS patients with SIBO does not necessarily depend upon reversal of the SIBO, as assessed in luminal secretions, cast doubt as to the importance of luminal SIBO in the pathophysiology of IBS symptoms, at least in younger subjects. Comparable studies have not been performed in elderly IBS patients with luminal SIBO. Similarly, the pathophysiological relevance of any disturbances of duodenal mucosa-associated microbiota in patients with IBS, including the reduced levels of *B. catenulatum* and increased levels of *Pseudomonas aeruginosa* levels so far demonstrated by culture-independent means, remains to be determined.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

# Irritable bowel syndrome: Emerging paradigm in pathophysiology

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## Abstract

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorders, characterized by abdominal pain, bloating, and changes in bowel habits. These symptoms cannot be explained by structural abnormalities and there is no specific laboratory test or biomarker for IBS. Therefore, IBS is classified as a functional disorder with diagnosis dependent on the history taking about manifested symptoms and careful physical examination. Although a great deal of research has been carried out in this area, the pathophysiology of IBS is complex and not completely understood. Multiple factors are thought to contribute to the symptoms in IBS patients; altered gastrointestinal motility, visceral hypersensitivity, and the brain-gut interaction are important classical concepts in IBS pathophysiology. New areas of research in this arena include inflammation, postinfectious low-grade inflammation, genetic and immunologic factors, an altered microbiota, dietary factors, and enteroendocrine cells. These emerging studies have not shown consistent results, provoking controversy in the IBS field. However, certain lines of evidence suggest that these mechanisms are important at least a subset of IBS patients, confirming that IBS symptoms cannot be explained by a single etiological mechanism. Therefore, it is important to keep in mind that IBS requires a more holistic approach to determining effective treatment and understanding the underlying mechanisms.



**Key words:** Pathophysiology; Irritable bowel syndrome; Inflammation; Immunologic; Genetics; Microbiota; Diet; Enteroendocrine cell

**Core tip:** In recent years, several novel mechanisms of irritable bowel syndrome (IBS) that likely relate to previously established IBS theories have been identified. Inflammation and postinfectious low-grade inflammation are emerging areas requiring clarification with regard to IBS pathophysiology. Immunological and genetic predisposition along with altered microbiota are critical in IBS development, while several dietary factors and enteroendocrine cells may also play roles in this syndrome. However, none of these accounts for the full repertoire of IBS symptoms, and the pathophysiology of this condition is not fully understood.

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## INTRODUCTION

Irritable bowel syndrome is a functional gastrointestinal disorder that manifests symptoms of recurrent abdominal pain associated with changes in bowel habit without

organic abnormalities[1], and its prevalence ranges from 5% to 15%[2]. According to the Rome Ⅲ Diagnostic Criteria, irritable bowel syndrome (IBS) is defined as a syndrome with recurrent abdominal pain or discomfort occurring at least 3 d per month over a 3-mo span. It is associated with two or more of the following characteristics: (1) improvement with defecation; (2) change in stool frequency with onset; and (3) change in stool form with onset[3]. Many studies in IBS pathophysiology over the past decades have focused on colonic dysmotility, visceral hypersensitivity, and the brain-gut interaction. Recently, however, other mechanisms have been actively studied, including inflammation[4], post-infectious low-grade inflammation[5], immunologic factors[6], altered microbiota[7], dietary factors[8] and enteroendocrine cells[9]. However, evidence regarding their roles in IBS remains controversial. Recently, the definition of IBS has been challenged by growing evidence of organic abnormalities in patients who satisfy the Rome criteria for IBS[10,11]. Due to these new paradigms, IBS may no longer classify as an absolute functional disorder. In this article, we briefly summarize the classical concepts and follow with a discussion of the recent research pertaining to the new models of IBS pathophysiology. Better understanding of these emerging paradigms will aid the diagnosis and management of IBS.

## CLASSICAL CONCEPTS IN THE PATHOPHYSIOLOGY OF IBS

### *Gastrointestinal dysmotility*

Gastrointestinal dysmotility is recognized as one of the primary pathophysiological mechanisms in IBS, but it does not fully correlate with symptomatic bowel disturbances. Colonic motor activity in healthy subjects mainly consists of non-propagating and sporadic contractions and progression of intestinal contents by propagating movements termed high-amplitude propagated contractions (HAPCs)[12-14]. The frequent occurrence of HAPCs in IBS patients may explain the frequent bowel movements that cause diarrhea in diarrhea-predominant IBS (D-IBS)[15,16], whereas HAPCs are rarer in patients with constipation-predominant IBS (C-IBS)[17]. Colonic transit is generally accelerated in D-IBS and delayed in C-IBS according to several studies; however, reports on the relationship between colonic motility and IBS subtypes are inconsistent[18]. In one survey, 70% of C-IBS and 50% of D-IBS patients noted the feeling of incomplete evacuation[19]. In contrast, more recent data provided evidence that pelvic floor dyssynergia (PFD) causes symptoms characteristic of non-diarrhea predominant IBS (non-D IBS), including straining, incomplete evacuation, blockage, digitation, and anal pain, suggesting that anorectal function tests should be considered in patients with non-D IBS and PFD symptoms[20].

### *Visceral hypersensitivity*

According to the classical concepts, IBS is caused by visceral hypersensitivity resulting in abdominal pain or discomfort and gastrointestinal motor disorder, which lead to alterations in defecation patterns; *i.e.*, diarrhea or constipation. Numerous studies have demonstrated the link between IBS and increased intestinal sensitivity[21]. Rectal hypersensitivity was proposed as a marker for IBS, and rectal sensory thresholds measured by rectal barostat testing were lower in IBS patients compared to healthy controls after rectal distention[22]. Most research so far has focused on colonic sensitivity[23,24] but hypersensitivity has also been observed in the esophagus[25], stomach[26] and small intestine[27] with IBS. Many studies have shown visceral sensitivity in IBS to correlate with stress[28] and food intake[29]. Colorectal sensitivity is attenuated in IBS patients after intake of a meal[30,31], and the visceral stimulus is significantly higher during stress in IBS patients than in healthy controls[32,33]. Therefore, visceral hypersensitivity is considered to be the conglomeration of peripheral and central processes[34], and its determinants are considered to be a combination of intrinsic and environmental factors.

### *Brain-gut interaction*

Alterations in the brain-gut axis are a new concept in IBS pathophysiology. Environmental, cognitive, and emotional states can affect intestinal sensory perception[35,36]. Corticotropin-releasing hormone (CRH) is a major mediator of stress responses in the brain-gut axis, affecting the functions of both the brain and the gut[37,38]. Intravenous administration of CRH exacerbated colonic motility[39], while peripheral administration of a CRH antagonist blocked the stress-induced increase in colonic motility, visceral perception, and negative mood[40]. Several studies have demonstrated brain-gut interactions using brain imaging. For example, Hamaguchi *et al*[41] showed that distention of the descending colon activated portions of the brain that are highly related to pain recognition and emotion. Mayer *et al*[42] reported that IBS patients exhibit increased activation of brain regions that potentially correspond to the perception of rectal distension. Finally, Mertz *et al*[43] showed differences in activation of brain regions in response to a painful rectal stimulus in IBS patients compared to controls.

## INFLAMMATION

Recent evidence supports a role for inflammation in IBS pathophysiology and generation of IBS symptoms in a subset of patients. Chadwick *et al*[44] performed studies of colonoscopic biopsy specimens from patients meeting the Rome criteria for clinical diagnosis of IBS. Immunohistological assessment showed an increased number of activated immunocompetent cells, including T-lymphocytes, neutrophils, and mast cells in the intestinal mucosa, suggesting a role for the mucosal immune system in pathogenesis. Subsequent studies demonstrated an increased frequency of several surrogate markers for inflammation in IBS patients, the most con-

sistent finding being an increased number of mast cells in the gastrointestinal (GI) tracts of IBS patients[4,45-47]. Mast cells are associated with wound healing, defense against pathogens, and hypersensitivity in GI mucosa. They degranulate to release inflammatory and immune mediators, which cause the recruitment of other inflammatory cells into the GI mucosa. Several studies have indicated that increased mast cells in IBS patients may correlate with certain symptoms of IBS, such as bloating and abdominal pain[46,48]. Another finding is the presence of activated T-lymphocytes in mucosal biopsy specimens from IBS patients[4,46,49]. Several studies have demonstrated an increase in the infiltration of lymphocytes in the myenteric plexus of patients compared to healthy controls[46,47,50]. Furthermore, patients with IBS have more activated T-cells in their colonic biopsies and blood samples[51]. T lymphocytes are involved in adaptive immunity and have multiple functions, such as the activation of B lymphocytes and macrophages and the destruction of infected host cells[52]. In addition, enhanced expression of proinflammatory cytokines in peripheral blood mononuclear cells[53] and serum[54] may confer a predisposition to immune activation in patients with IBS. In the following section, we will review the data supporting the role of inflammatory and proinflammatory cytokines in IBS.

IBS-like symptoms seen in ulcerative colitis (UC) patients during the remission phase appear to involve inflammation[55-57]. It is assumed that chronic inflammation in the colon during the remission phase, associated with altered sensory and motor functioning, can lead to IBS-like symptoms[58,59]. Fecal calprotectin was significantly higher in IBD patients displaying IBS-like symptoms than those lacking IBS-like symptoms, indicating the presence of occult inflammation in the former[55]. One group reported elevated levels of beta-defensin 2 peptides (HBD-2) in fecal fluid derived from IBS patients[60]. HBD-2 is an antimicrobial peptide recently implicated in the pathogenesis of inflammatory bowel disease[61]. These results suggest an activation of the mucosal innate defense system toward a proinflammatory response in IBS patients without macroscopic signs of inflammation.

There is also evidence of microscopic inflammation in IBS. In our previous study, conducted in 42 IBS patients diagnosed by the Rome Ⅱ criteria, the microscopic findings of mucosal hyperplasia, lymphocyte aggregation, and increased eosinophil counts were more frequently observed in the IBS group than the control group. Microscopic colitis does not appear to be associated with IBS symptoms[62]. A study in Malaysia also identified microscopic inflammations in D-IBS subjects that did not meet the criteria for classical microscopic colitis. In this study, the most common pathological findings were mixed chronic and acute inflammatory cells, lymphocytes, plasma cells and neutrophils[63]. IBS onset following an episode of gastroenteritis [post-infectious IBS (PI-IBS) is indicative of a role for inflammation in the pathogenesis of IBS (discussed below)]. Although large amount of research focusing on inflammation in the pathophysiology of IBS, as discussed in this section, this concept should be studied further to develop a potential future therapy for IBS.

## POST-INFECTIOUS LOW-GRADE INFLAMMATION

Recently, numerous studies indicated that bacteriologically confirmed gastroenteritis is critical in the pathogenesis of IBS[5,64,65]. Also called post-infectious IBS (PI-IBS), first proposed by Stewart[66] in 1950, this is a case where IBS symptoms emerge in a patient - who has not previously met the Rome criteria for IBS - following an infectious illness characterized by two or more of the following: fever, vomiting, diarrhea, or a positive bacterial stool culture[67]. Most patients with infectious gastroenteritis recover in a few days, but approximately 10% of patients experience persistent symptoms (*e.g.*, abdominal pain or diarrhea) that progress to IBS[64]. In the meta-analysis by Thabane *et al*[65], the odds of developing IBS increased six- to seven-fold in patients with an episode of acute gastroenteritis. The mechanisms of PI-IBS are still not clear, yet studies have indicated that inflammation[68], genetic polymorphisms in genes associated with immune responses to infectious pathogens[69], and immune functioning[70] may contribute to the occurrence of PI-IBS.

Low-grade inflammation is recognized as the main pathophysiology of PI-IBS. El-Salhy *et al*[71] reported that rectal biopsy specimens taken from patient after *Campylobacter* gastroenteritis showed increases in leucocytes, lymphocytes, mast cells and endocrine cells. Another study reported that 3 mo post-gastroenteritis, patients who had PI-IBS continued to increase their chronic inflammatory cell counts, while those in healthy controls returned to normal levels[72]. Furthermore, several studies demonstrated that intestinal mast cell infiltration and activation following an infection often resulted in mucosal inflammation and the development of PI-IBS[5,73]. Such findings support a relationship between mucosal inflammation and PI-IBS. Development of IBS following non-GI infection has also been reported[74], and other recent study found that viral and bacterial enteritis outbreaks can lead to PI-IBS in a considerable proportion of patients (13%)[75].

Several lines of evidence indicate that inflammation and immune cells play roles in the intestinal neuroendocrine system, which controls GI sensory-motor function[76]. Dunlop *et al*[77] identified an association between PI-IBS and the persistence of mucosal abnormalities, enterochromaffin cell (EC) hyperplasia, and increased mucosal permeability, including intestinal inflammation. Increased permeability facilitates transfer of antigens through the intestinal mucosa, which leads to inflammatory cascades characterized by increased immune cell numbers. Serotonin secretion from EC cells, which regulates the gut immune system, can be attenuated by the secretory products of immune cells[78,79].

There are reports of increased levels of the proinflammatory cytokines in plasma levels of PI-IBS patient[54] and significantly greater IL-1β mRNA expression in the rectal mucosa of patients with IBS symptoms following acute gastroenteritis, but not in asymptomatic control subjects[73,80]. Flagellin antibodies were observed more frequently in patients with PI-IBS, indicating that immune activation in response to luminal triggers plays a role in the development of IBS[81,82]. Flagellins are primary triggers of innate and adaptive immunity, thus driving pathogen-induced acute inflammation[83]. These observations suggest that inflammatory responses to infection, rather than the infective pathogen itself, are an important predisposition to the occurrence of PI-IBS.

## IMMUNOLOGIC AND GENETIC FACTORS

More recent data indicate an influence of genetics on the development of IBS. A survey of twins in Norway showed that the concordance for IBS in monozygotic twins was significantly higher than in dizygotic twins, providing robust evidence for the involvement of genetic factors in the etiology of IBS[84]. To date over 60 candidate genes have been reported as positively associated with IBS[85]. It should be noted that many of these studies had conflicting results; nevertheless, similar surrogate markers are being examined. Discrepancies may be due to differences in IBS subtypes of the study subjects, or in the processes by which the studies recruited their control groups, or in the laboratory methodologies used. However, it is noteworthy that many of these cases demonstrated genetics as a potential etiological factor. The representative genetic factors for IBS pathophysiology associate with inflammation, neurotransmitters, and bile acid synthesis.

### *Inflammation*

Transient mucosal inflammation is crucial for the manifestation of IBS, despite the original definition of this syndrome that implies the lack of signs of active inflammation[86]. According to the evidence, subsets of IBS patients share genetic susceptibility loci for inflammation. The relatively well-studied IBS gene is *TNFSF15*, which has been confirmed in genome-wide association studies to mediate mucosal inflammation in IBD[87]. In Crohn's disease, TNFSF15 is up-regulated with intestinal inflammation and functions in nuclear factor κB activation, potentiation of IL-2 signaling, and secretion of interferon gamma by T lymphocytes[88]. Three cohort studies performed in the United Kingdom[69], Sweden and the United States[89], and England[90] identified a significant association between TNFSF15 and IBS. Belmonte *et al*[91] provided further evidence for altered intestinal immune activation. Increased toll-like receptor (TLR) expression has previously been observed in IBD[92]. In this study, the expression of TLR2 and TLR4 differed significantly among the IBS subtypes. The increased TLR expression in mixed-type IBS patients provoked intracellular signaling pathways that resulted in increased expression of the mucosal proinflammatory cytokines IL-1 and IL-8[91]. Villani *et al*[93] suggested genetic risk factors for the development of PI-IBS based on a 2300-patient cohort in Walkerton, Ontario. They found that TLR9, IL-6, and CDH1 variants persisted as independent risk factors for PI-IBS. Similarly, Brint *et al*[94] reported elevated levels of TLR4 and TLR5 level in PI-IBS patients, supporting the involvement of the innate immune system leading to an inflammatory response.

Several studies identified specific genetic polymorphisms in proinflammatory cytokines, which have an influence on GI functions, motility, epithelial permeability, and visceral sensation[95-97]. TNF-alpha is produced by monocyte-derived activated macrophages, and this cytokine plays on important role in chronic inflammatory states such as IBD[98]. According to a study in the Netherlands, increased TNF-alpha levels were significantly more prevalent in IBS patients compared to healthy controls, while no such association was found for polymorphisms in the *IL-10* gene[6], an anti-inflammatory cytokine involved in the regulation of immune and inflammatory responses. Several studies identified that certain IBS patients may be genetically predisposed to decreased production of IL-10 and subsequent development of low-grade inflammatory manifestations of IBS[99]. In a study done in Mexico, the high IL-10 producer genotype is less prevalent in IBS patients than healthy controls[86]. However, in the abovementioned Netherlands study, *IL-10* genotypes were similarly distributed among patients with IBD compared to healthy controls[6]. In contrast, in Japanese subjects, the frequency of the IL-10 genotype was significantly higher in IBS-D and UC than that in controls[100]. Although IL-10 might be associated with susceptibility to IBS development, many important questions remain regarding this relationship.

### *Neurotransmitters and cytokines*

Among the single genetic polymorphisms associated with IBS, the role of the serotonin transporter (*SERT*) gene polymorphism (*SLC6A4*) has been relatively well explored in IBS. This polymorphism varys according to geographical region and ethnic population. In a meta-analysis, a genetic polymorphism in the gene region responsible for *SERT* activity was not associated with IBS[101]. However, subsequent studies reported inconsistent results. Kumar *et al*[102] did show that a *SLC6A4* polymorphism was significantly associated with IBS, and Wang *et al*[103] found that different *SERT* genotypes could influence *SLC6A4* promoter efficiency and *SERT* mRNA and protein expression in the colonic mucosa.

G proteins are expressed in all human cells and play a crucial role in signal transduction, particularly ligand-receptor interactions. The G protein is encoded by the *GNbeta3* gene. Although, *GNbeta3* polymorphisms have been linked to functional dyspepsia, such association was not observed with IBS[104,105]. However, Saito *et al*[106] reported a significant interaction between the *GNbeta3*

polymorphism and infection during IBS development, suggesting that IBS is a complex genetic disorder with both a genetic and environmental component for expression of symptoms.

Neuropeptide S (NPS) is a bioactive 20 amino acid peptide that selectively binds and activates the neuropeptide S receptor (NPSR1). NPSR1 induces the production of several neuropeptides, including cholecystokinin, vasoactive intestinal peptide, peptide YY, and somatostatin. NPSR1 variants are associated with gastrointestinal motor and sensory functions that are relevant to IBS[107].

The endocannabinoid system, involved in motility[108], sensation[109], secretion[110,111] and inflammatory[112,113] functions in the gastrointestinal tract, has been proposed as a mechanism in the development of IBS. The endocannabinoid anandamide is inactivated by the fatty acid amide hydrolase (FAAH), and single nucleotide polymorphisms (SNPs) in the *FAAH* gene (*C385A*) have been associated with accelerated colonic transit time in D-IBS[108].

### Genetic variation in bile acid synthesis

Genetic variation in the genes controlling bile acid synthesis may contribute to abnormal bowel pattern and symptoms in IBS. Bile acid malabsorption stimulates colonic motility and secretion and has been associated with D-IBS[114]. Hepatic bile acid synthesis is partially controlled by feedback inhibition *via* the fibroblast growth factor 19 (FGF19); FGF19 binds to the FGF receptor 4 and the co-receptor Klotho-beta (KLB), leading to suppression of the rate-limiting enzyme in bile acid synthesis[115]. Wong *et al*[116] reported that a SNP in the *KLB* gene (rs17618244), is associated with accelerated colonic transit in IBS-D. A previous study suggested that the G protein-coupled bile acid receptor 1 (GpBAR1/TGR5) is expressed in myenteric, cholinergic, nitrergic neurons in the colon and in the proximal small intestine, indicating that bile acids may alter intestinal and colonic motility[117]. Camilleri *et al*[118] demonstrated that variations in TGR5 might contribute to altered SBT and colonic transit in D-IBS patients.

## ALTERED INTESTINAL MICROBIOTA

The intestinal microbiota has recently been assumed to be an important predisposition factor for IBS. The most convincing evidence is that IBS can develop in predisposed persons who have experienced gastroenteritis. Other evidence indicates that bacteria may contribute to the pathophysiology of IBS, since luminal- and mucosa-associated microbiota can influence their host *via* immunomicrobial interactions[119]. In addition, small intestinal bacterial overgrowth (SIBO) has been implicated in a subset of IBS patients.

Earlier studies found that the intestinal microbiota in IBS patients differs from that in healthy individuals, with a decrease in the *Bifidobacterium* spp. population and an increase in the *Enterobacter* population being the most consistent findings[120,121]. In a study using real-time PCR assays, results included significantly lower counts of *Lactobacilli* in D-IBS than C-IBS specimens, lower counts of *Bifidobacterium* spp. in D-IBS than the other groups, and significantly higher counts of *Veillonella* spp. counts in the C-IBS group than healthy controls[122]. High-throughput analysis of 16S ribosomal RNA gene cloning and sequencing identified that the fecal microbiota is considerably altered in IBS, as IBS patients have lower *Lactobacillus* and *Bifidobacterium* spp. counts than healthy subjects[123]. Subsequent molecular studies confirmed that IBS patients have fecal microbiota differing from normal subjects[124-126]. Results regarding the intestinal microbiota in IBS are difficult to interpret due to the heterogeneity of the conditions and the observation that alterations of the intestinal microbiota may not be consistent across each subtype of IBS. Furthermore, the precise role of the luminal *vs* the mucosal-associated microbiota in IBS remains uncertain. Nevertheless, previous evidence consistently showed differences in the bacterial composition of feces between IBS and normal controls. Changes in the intestinal flora might result in the proliferation of species that produce more gas[127,128] during the development of IBS symptoms that bring about gas-induced distension. The direct effects of bacterial production on colonic contractility[129], intestinal myoelectrical activity[130], and pain response[131,132] have been identified in several *in vitro* studies. Also, a role for the microbiota in the induction of IBS symptoms is supported by the findings that probiotics improve flatulence and abdominal distension[133,134] and that rifaximin provides significant improvements in IBS symptoms, including bloating, abdominal pain, and loose or watery stools[135].

A growing body of research implicates SIBO in the symptoms of IBS, but this issue remains under debate. SIBO proved to be more prevalent in patients with IBS patients[136-138], and its eradication with antibiotics relieved the symptoms of IBS[139-142]. The presence of SIBO might be associated with abnormalities in small intestinal motor function. Pimentel *et al*[143] found that patients with IBS and SIBO experience few, if any, phase Ⅲ events during short-term manometric measurements compared to controls. In contrast, Posserud *et al*[144] performed intestinal manometry and culturing of intestinal aspirates taken from IBS and control groups, found that IBS subjects have fewer Major Migrating Complex phase Ⅲ events compared to patients without SIBO. However, there were no differences in other motility parameters, and no correlation between bacterial numbers and the pattern of IBS symptoms was detected. SIBO is typically diagnosed *via* indirect methods, such as positive early glucose or lactulose breath tests, and the accuracy of these methods is arguable. These diagnostic limitations have resulted in wide range of reports for SIBO prevalence (10% to 84%) in patients with IBS[145,146]. Regardless, slightly elevated intestinal bacterial numbers are inarguably more prevalent in IBS patients, and so further studies of this area are required.

## DIETARY FACTORS

Although the "response to food" is not included in the diagnostic criteria for IBS, most patients claim their symptoms are triggered by certain foods, which are then avoided to alleviate symptoms[147,148]. Many researchers have focused on the role of diet in IBS in recent years. Also, guidance on diet management for patients with IBS has been revealed as improving their quality of life and symptoms[149,150]. The sensory component of the gastro-colonic reflex following nutrient intake is exaggerated in IBS patients[30], and IBS patients with intraluminal lipids exhibited impaired intestinal gas clearance because of an upregulated reflex inhibition in small bowel transit[151]. One study demonstrated that postprandial GI disorders in IBS patients might be associated with cellular immune function along the neuroendocrine-immune axis[152]. Furthermore, altered autonomic responses after a meal might cause exacerbated postprandial symptoms in IBS patients[153].

### *Food allergy and intolerance*

Many IBS patients report that their symptoms are associated with specific foods; thus, the possibility of food allergies causing IBS symptoms has been proposed. Food allergy/hypersensitivity is defined as an allergic response in susceptible individuals following ingestion of a specific food (*e.g.*, cow's milk, peanuts, soybeans)[154,155]. However, there is little evidence that food allergies play a role in IBS. Several studies have reported that fructose-sorbitol malabsorption frequently occurs in IBS patients, but the results were similar in healthy volunteers; further, the response to a low lactose diet was disappointingly low in IBS patients experiencing lactose malabsorption, indicating a lack of obvious association between food allergy and IBS[156,157]. Several lines of evidence indicate that an altered immune response and inflammation may be involved in food hypersensitivity in IBS patients. There are reports of IgG-mediated food hypersensitivity and improved IBS symptoms when patients are placed on elimination diets[158-160]. Carroccio *et al*[161,162] demonstrated in IBS patients with food hypersensitivity an activation of serum basophils after stimulation with food antigens and increased levels of fecal eosinophil cationic proteins and tryptases. However, further investigations are necessary to validate the accuracy of the methods used in these studies before any claims can be made.

Food intolerances are defined as non-toxic and non-immune-mediated adverse reactions to food or to the presence of pharmacological agents within food, including histamines, sulfates, monosodium glutamate, serotonin, norepinephrine and tyramine[163]. Food intolerance is a possible factor underlying the pathogenesis of IBS, according to the finding that symptoms improved with an elimination diet[164]. However, subsequent studies showed little benefit from these diets[165,166]. Although specific food intolerances in IBS have been explored through patient questionnaires[167,168], the role of food intolerance in IBS remains questionable due to the lack of a reliable methodology and well-designed trials. Well-designed studies with standardized protocols are thus necessary.

### *Poorly absorbed nutrients*

A recently proposed mechanism by which dietary factors might contribute to IBS symptoms suggests that poor absorption of nutrients influence GI function and sensation through osmotic actions and colonic fermentation[163]. Short-chain carbohydrates, such as fructose and dietary starch, are poorly absorbed, causing a number of ingested carbohydrates to enter the distal small bowel and colon. Consequently, these provide substrates for short-chain fatty acid (SCFA) generation by bacterial fermentation and increase the osmotic pressure[169]. The short-chain carbohydrates called Fermentable Oligo-saccharides, Di-saccharides, Mono-saccharides And Polyols (FODMAPs) contribute to IBS; however, IBS symptoms in such cases are triggered by luminal distension that induces abdominal pain, bloating, flatus, and altered bowel habits[170]. A number of studies suggesting effects of dietary manipulation, particularly elimination of FODMAPs, further support the importance of poor nutrient absorption in the development of IBS symptoms[171,172]. In addition, fecal SCFA were increased in D-IBS[173]. SCFA stimulate colonic transit and motility *via* intraluminal release of 5-hydroxytryptamine (5-HT)[174] and high-amplitude propagated colonic contractions[175], according to *in vivo* studies.

Limited data also suggest that changes in intestinal microbiota may be relevant for fermentation of non-absorbable nutrients[169]. Tana *et al*[176] showed that an altered GI microbiota contributes to the higher levels of SCFA and abdominal symptoms in IBS. In addition, many studies have explored the effect of dietary fiber on IBS symptoms. Although dietary fiber has commonly been a standard recommendation for patients with IBS[177], some evidence suggests that it may aggravate symptoms of IBS, such as flatulence, bloating and abdominal pain[76,127,128]. In a recent meta-analysis, patients administered fiber in their diets had persistent or unimproved symptoms compared to control groups that ingested placeboes or lower fiber diets[177]. Some investigators suggest that insoluble fiber intake does not significantly improve IBS symptoms, whereas soluble fiber intake can effectively improve overall IBS symptoms[178,179]. These results suggest that not all types of fiber are equally influential on IBS.

### *Gluten intolerance*

Patients with celiac disease (CD) often experience IBS-like symptoms[180,181]. Therefore, it has been proposed that IBS patients should be routinely examined for CD[163,181]. Certain evidence suggests that dietary gluten intolerance also occurs in patients with IBS, and those whose symptoms improve with such diets may have a genetic susceptibility to gluten[182,183]. However, two groups of investigators recently published contrasting

results. Vazquez-Roque *et al*[184] conducted randomized controlled trials in D-IBS patients consuming gluten-free diets *vs* gluten-containing diets. The group consuming gluten showed increased stool frequency, small intestinal permeability, and reduced mRNA expression of tight-junction proteins in bowel mucosa compared to the patients consuming the gluten-free diet. However, Biesiekierski *et al*[185] reported that gluten might be not be a specific trigger of GI symptoms in IBS patients, as most patients' symptoms were not exacerbated with gluten exposure; there was no evidence of specific or dose-dependent effects of gluten in the expression of serum and fecal markers of intestinal inflammation/injury and immune activation. Further studies are being conducted to determine the role of gluten intolerance in IBS.

## ENTEROENDOCRINE CELLS

Abnormalities in neuroendocrine peptides and amines derived from enteroendocrine cells can cause disturbances in digestion, GI motility and sensation in IBS patients[76,186]. These abnormalities are stimulated by gut luminal content, contributing to the development of symptoms in IBS[187]. Enteroendocrine cells release various bioactive substances, including gastrin, secretin, stomatostatin, cholecystokinin, chromogranins and serotonin[79]. Enterochromaffin cells, which are scattered throughout the GI mucosa, are the dominant type of enteroendocrine cells; they synthesize, store, and release serotonin in response to luminal stimuli[187]. Serotonin affects motility, sensation, and secretion in the gut through the activation of receptors present on enteric nerves and sensory afferents[188]. Studies demonstrated an increase in the release of serotonin in patients with D-IBS[189,190] and PI-IBS[191], while impaired release was found in patients with C-IBS[191,192]. The increased release of 5-HT triggered by luminal stimuli activates immune cells supporting the role of 5-HT in gut inflammation[79].

In addition to serotonin, enteroendocrine cells release chromogranin and secretogranin, which can influence several GI functions, such as immune modulation and inflammation[79]. El-Salhy *et al*[193-195] showed that decreased density of chromogranin A (CgA)-containing cells was found in duodenum, terminal ileum and colonic mucosa of IBS patients. Whereas, other studies demonstrated that serum CgA levels increase in IBS patients[196,197]. Recently, Ohman *et al*[9] showed that IBS patients with rapid colonic transit have higher levels of fecal CgA, secretogranin (Sg) Ⅱ, and SgⅢ, but lower levels of chromogranin B, compared to healthy subjects. Based on the data, granins could serve as useful biomarkers of IBS; however, the role of granins in IBS has not been revealed.

## CONCLUSION

There is abundant evidence supporting the claim that IBS should no longer be considered an absolute idiopathic functional disease. In recent years, attention has been directed towards the role of inflammation, gut microbiota, immunity, genetics, dietary factors, and enteroendocrine cells. As a result, IBS is regarded as a multifactorial condition that affects individuals differentially. Understanding these mechanisms will be useful for the development of a more specific, individualized treatment strategy and for the clinical management of IBD patients.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

# Unraveling the ties between irritable bowel syndrome and intestinal microbiota

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## Abstract

Irritable bowel syndrome (IBS) is the most prevalent functional gastrointestinal disorder. It is a multifactorial disorder. Intestinal microbiota may cause the pathogenesis of IBS by contributing to abnormal gastrointestinal motility, low-grade inflammation, visceral hypersensitivity, communication in the gut-brain axis, and so on. Previous attempts to identify the intestinal microbiota composition in IBS patients have yielded inconsistent and occasionally contradictory results. This inconsistency may be due to the differences in the molecular techniques employed, the sample collection and handling methods, use of single samples that are not linked to fluctuating symptoms, or other factors such as patients' diets and phenotypic characterizations. Despite these difficulties, previous studies found that the intestinal microbiota in some IBS patients was completely different from that in healthy controls, and there does appear to be a consistent theme of *Firmicutes* enrichment and reduced abundance of *Bacteroides*. Based on the differences in intestinal microbiota composition, many studies have addressed the roles of microbiota-targeted treatments, such as antibiotics and probiotics, in alleviating certain symptoms of IBS. This review summarizes the current knowledge of the associations between intestinal microbiota and IBS as well as the possible modes of action of intestinal microbiota in the pathogenesis of IBS. Improving the current level of understanding of host-microbiota interactions in IBS is important not only for determining the role of intestinal microbiota in IBS pathogenesis but also for therapeutic modulation of the microbiota.



**Key words:** Irritable bowel syndrome; Intestinal microbiota; Dysbiosis; Antibiotics; Probiotics

**Core tip:** The intestinal microbiota is altered in some Irritable bowel syndrome (IBS) patients, and the symptoms of IBS can be alleviated by treatments that target the microbiota. Over the past several years, many studies have attempted to identify the intestinal microbiota composition in IBS patients and intestinal dysbiosis in IBS is characterized by *Firmicutes* enrichment and reduced abundance of *Bacteroides*. Based on the differences in intestinal microbiota composition, the roles of microbiota-targeted treatments, such as antibiotics and probiotics, were investigated in alleviating certain symptoms of IBS.

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## INTRODUCTION

Irritable bowel syndrome (IBS) is characterized by abdominal discomfort, bloating, and disturbed defecation in the absence of any identifiable abnormalities indicative of organic gastrointestinal disease[1]. IBS is the most commonly diagnosed gastrointestinal disorder, and it accounts for about 30% of all referrals to gastroenterologists[2]. In the general population worldwide, its prevalence has been reported to range from 5% to 25%[1,3-6]. IBS worsens patients' quality of life significantly, and both patients and healthcare systems incur huge costs toward its treatment[6]. Several treatments and therapies help alleviate the symptoms of IBS; however, they do not cure this condition. Thus, the chronic nature of IBS and the challenge of controlling its symptoms can be frustrating for both patients and healthcare providers[1,2].

IBS is a multifactorial disorder, and its underlying pathophysiology is unclear[1]. Therapeutic strategies have traditionally focused on alterations in gastrointestinal motility and visceral hypersensitivity influenced heavily by stress[7]. However, some drugs that target gastrointestinal motility and visceral hypersensitivity, such as antidepressants, alosetron, and tegaserods, have only a narrow therapeutic window, limiting their clinical application, especially in mild cases of IBS[8]. Therefore, studying the pathophysiology of IBS is important, especially in light of the possibility of developing targeted therapies. More recent studies have focused on the role of altered intestinal microbiota[7,9,10].

Since prospective studies have demonstrated that 3%-36% of enteric infections lead to new, persistent IBS symptoms[10], the concept that gut microbes play an important role in the pathogenesis of IBS was confirmed. Recent studies have demonstrated an unimagined level of complexity in human intestinal microbiota, with thousands of phylotypes, 80% of which remain uncultured[11]. The introduction of culture-independent techniques for studying intestinal microbiota has increased our understanding of the role of intestinal microbiota in human diseases, and emerging studies have demonstrated changes in intestinal microbiota in patients with IBS[12-14]. The restoration of altered intestinal microbiota may be a new therapeutic option for treating IBS[15]. Previous randomized controlled trials (RCTs) have documented that the symptoms of IBS can be improved by treatments that target the microbiota, such as antibiotics and probiotics[7]. Herein, the evidence of associations between the intestinal microbiota composition and IBS is reviewed, and the possible roles of specific microbial groups in IBS management are discussed in light of the most recent findings.

## HUMAN INTESTINAL MICROBIOTA

The human body is inhabited by a complex community of microbes that are collectively referred to as human microbiota. The human intestinal microbiota constitutes a complex and metabolically active ecosystem that is now well recognized for its impact on human health and disease[16]. It is estimated that the human microbiota number more than $10^{14}$ cells, which exceeds the number of human cells in our bodies[7]. The microbiota is taxonomically classified according to the traditional biological nomenclature (phylum-class-order-family-genus-species), and currently, more than 50 bacterial phyla have been described, of which 10 inhabit the colon and three bacterial phyla, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* predominate[17]. Genotypic sequencing studies based on the 16S ribosomal RNA (16S rRNA)-encoding gene have been used for demonstrating that the human gastrointestinal tract can be populated by any of 1000-1150 different species[18]. Despite this diversity, a core of 18 species was found in all individuals, and 57 were found in 90% of individuals, indicating considerable dominance and inter-individual stability of these species across humans[18]. Faith *et al*[19] analyzed the fecal microbiota of 37 individuals and found that, on average, 60% of the bacterial strains present remained stable for up to 5 years; many were estimated to remain stable for decades.

Recent analyses of human-associated bacterial diversity have tried to categorize individuals into "enterotypes" based on the abundances of key bacterial genera in the intestinal microbiota[20]. Arumugam *et al*[21] reported that a set of 22 Sanger-sequenced European fecal metagenomes from Danish, French, Italian, and Spanish individuals was shown to fit into three distinct clusters (enterotypes), each characterized by variations in the numbers of *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), and *Ruminococcus* (enterotype 3). Recent meta-analysis including the 16S rRNA sequences and whole genome shotgun sequences from the Human Microbiome Project, Metagenomics of the Human Intestinal Tract consortium, and additional studies yielded only bimodal distributions of *Bacteroides* abundances in gut samples[20]. Enterotype identification depends not only on the structure of the data but also on the methods used for identifying clustering strength[20].

The diversity of intestinal microbiota within and among individuals is strongly influenced by factors such as age, diet, and diseases[9]. In a large cross-sectional study of an elderly population using pyrosequencing, the intestinal microbiota of the elderly subjects was found to be different from that of younger adults, with higher *Bacteroides* and *Clostridia cluster IV*, as well as some signature sequences that were present only in older people[22]. The impact of food intake on the microbiota is being explored. Habitual long-term diet has been shown to be strongly associated with enterotype, with protein/animal fat being associated with *Bacteroides* abundances and carbohydrate being associated with *Prevotella* abundances[23]. In a comparative study in children from urban Europe and rural Africa, rural African children showed significant enrichment in *Bacteroidetes* and depletion in *Firmicutes*, with a unique abundance of bacteria from the genus *Prevotella* and *Xylanibacter*, which are known to contain a set of bacterial genes for cellulose and xylan hydrolysis and were completely lacking in the urban European children[24]. In addition, obese individuals show an increase in

*Firmicutes* and a decrease in *Bacteroidetes*, probably owing partly to differences in diets[25]. Furthermore, manipulation of dietary macronutrients in gnotobiotic mice was shown to account for the majority of the change in their microbiota[26]. Moreover, many dietary prebiotics including oligo-fructose[27], lactulose[28], lupin kernel[29], inulin-containing juices[30], and arabinoxylan-oligosaccharides[31] significantly alter human fecal microbiota.

Characterization of intestinal microbiota, however, has been limited to Western people. A recent study investigated the overall intestinal microbiota composition of 20 Koreans using pyrosequencing[32]. Microbial communities were dominated by five previously identified phyla: *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, and *Proteobacteria*. Cluster analysis showed that the species composition of intestinal microbiota was host-specific and stable over the duration of the test period, but the relative abundance of each species varied among individuals. The results were compared with those of individuals from the United States, China, and Japan, and it was found that human intestinal microbiota differed among countries, but tended to vary less among individual Koreans. The gut microbial composition may be related to the internal and external characteristics of each country member, such as host genetics and dietary patterns[32].

## INTESTINAL MICROBIOTA COMPOSITION OF IBS PATIENTS

Numerous diseases have been associated with alterations in the microbiota, which are referred to as dysbiosis, ranging from systemic disorders such as obesity and diabetes to gastrointestinal disorders such as IBS[9,33]. The major physiological and immunological functions of the gut cannot be carried out in the absence of the intestinal microbiota[34,35]. The differences in the intestinal microbiota of IBS patients and those of healthy controls have been studied. A previous study that used cultures of fecal material obtained from patients with IBS reported decreased fecal *Lactobacilli* and *Bifidobacteria*, increased facultative bacteria dominated by *Streptococci* and *Escherichia coli*, as well as higher counts of anaerobic organisms such as *Clostridium*[36,37]. Traditional microbiology studies and microbial genome sequencing relied upon cultivated clonal cultures. Such culture-based assessment of fecal microbiota is cheap, widely available, and easy to use, but it grossly underestimates fecal populations because more than 80% of the bacteria in the human intestinal tract cannot be cultured using currently available methods[38].

A revolution in DNA sequencing technologies would be to define genetic material recovered directly from environmental samples. Metagenomics refers to culture-independent and sequencing-based studies of the collective set of genomes of mixed microbial communities (metagenomes) with the aim of exploring their compositional and functional characteristics[39]. In 1977, Woese *et al*[40] identified 16S rRNA, which is a component of the 30S small subunit of prokaryotic ribosomes, having relatively short gene sequences and highly conserved primer binding sites and containing hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. Since then, the molecular profiling of bacterial communities *via* 16S rRNA-gene based approaches such as terminal restriction fragment length polymorphism, PCR temperature/denaturing gradient gel electrophoresis, and fluorescent *in situ* hybridization, has been performed[41]. In the last decade, Sanger sequencing was used for generating data in most microbial genomics and metagenomics sequencing projects; however, recent advances in molecular biology have resulted in the application of DNA microarrays and next-generation sequencing (NGS) technologies for studying complex intestinal microbiota. DNA microarrays comprising hundreds or thousands of DNA fragments arrayed on small glass slides were originally developed for gene expression profiling. These were subsequently applied to the study of different aspects of microbial ecology, including total microbial diversity and a range of biogeochemical functions[42]. Alternatively, NGS approaches, including pyrosequencing (introduced by 454 Life Sciences, Inc.) as well as other platforms such as Solexa (Illumina, Inc.) and SOLiD (ABI, Inc.), offer rapid and highly parallel sequencing of many DNA fragments from complex samples or transcriptomes[39]. Pyrosequencing is particularly suited to microbial ecology studies because of its relatively long read length compared with other NGS technologies platforms, and it has therefore been widely adopted by microbial ecology researchers; other platforms have also been recently adopted in this field[42]. Table 1 lists the advantages and disadvantages of the principal techniques used for characterizing intestinal microbiota.

Studies using culture-independent molecular-based techniques revealed changes in the intestinal microbiota composition in IBS patients compared with those of healthy controls. Thus far, the results of studies on the intestinal microbiota of IBS patients are inconsistent and occasionally, contradictory (Table 1). This inconsistency in results may be ascribed to several reasons, including differences among the various molecular techniques employed, sample collection and handling methods, , as well as definitions of IBS and IBS subtypes[16]. Table 2 lists the advantages and disadvantages of the principal techniques used for characterizing intestinal microbiota. In studying human intestinal microbiota, classical approaches suffer from individual advantages and limitations[7,16]. NSG and phylogenic metagenomics update the bacterial community profiles of patients with IBS. The sample collection method can influence the intestinal microbiota composition. Namely, fecal samples show distal colonic luminal microbiota, whereas biopsy samples show only mucosa-attached microbiota. Although feces or fecal swabs are the most convenient samples, they do not accurately reflect the microbiota composition or activities in the proximal colon. Colon biopsies also do not represent the microbiota in its physiologic state because extensive colon preparation for cleaning intestinal contents removes

**Table 1 Summary of molecular studies of intestinal microbiota in irritable bowel syndrome**

| Ref. | Ethnicity | IBS patients, *n* | Mean age (range), yr | Male gender, *n* | IBS subtype | | | Controls, *n* | Sample | Method | Changes in intestinal microbiota composition in IBS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | IBS-C | IBS-D | IBS-M | | | | |
| Malinen *et al*[83] 2005 | Finland | 27 | 46.5 (20-65) | 7 | 9 | 12 | 6 | 22 (age, gender matching) | Feces | qPCR covering bacteria 300 bacterial species | IBS-D: ↓ lactobacillus spp.<br>IBS-C: ↑ veillonella spp.<br>Overall IBS: ↓ clostridium coccoides subgroup, Bifidobacterium catenulatum group |
| Mättö *et al*[46] 2005 | Finland | 26 | 46 (20-65) | 7 | 9 | 12 | 5 | 25 (age, gender matching) | Feces | Culture, PCR-DGGE | Temporal instability in the bacterial population<br>↑ coliform bacteria<br>↑ aerob:anaerob ratio |
| Maukonen *et al*[84] 2006 | Finland | 24 | 45 (24–64) | 5 | 6 | 7 | 3 | 16 | Feces | PCR-DGGE, Transcript analysis with the aid of affinity capture for Clostridial groups | Temporal instability in the bacterial population<br>IBS-C: ↓ clostridium coccoides-Eubacterium rectale group |
| Kassinen *et al*[43] 2007 | Finland | 24 | 47.3 (21–65) | 5 | 8 | 10 | 6 | 23 (age, gender matching) | Feces | GC-profiling + high-throughput *16S rRNA* gene sequencing of 3753 clones | Coverage of the clone libraries of IBS subtypes and control subjects differed |
| Kerckhoffs *et al*[85] 2009 | The Netherlands | 41 | 42 ± 2.12 | 12 | 11 | 11 | 16 | 26 | Feces, Duodenal mucosa | FISH, qPCR | ↓ bifidobacterium catenulatum |
| Krogius-Kurikka *et al*[86] 2009 | Finland | 10 | 46.5 | 4 | 0 | 10 | 0 | 22 | Feces | G + C (%G + C) -based profiling and fractioning combined with *16S rRNA* gene clone library sequencing of 3267 clones | ↑ proteobacteria<br>↑ firmicutes<br>↓ actinobacteria<br>↓ bacteroidetes |
| Lyra *et al*[87] 2009 | Finland | 20 | IBS-D: 43.6 (26-60), IBS-C: 48.6 (24-64), IBS-M: 50.8 (31-62) | 6 | 8 | 8 | 4 | 15 | Feces | qPCR | IBS-D: ↑ ruminococcus torques, ↓ clostridium thermosuccinogenes<br>IBS-C: ↑ ruminococcus bromii-like<br>IBS-M: ↓ ruminococcus torques, ↑ clostridium *thermosuccinogenes* |
| Tana *et al*[88] 2010 | Japan | 26 | 21.7 ± 2.0 | 13 | 11 | 8 | 7 | 26 (age, gender matching) | Feces | Culture, qPCR | ↑ veillonella spp.<br>↑ lactobacillus spp. |
| Codling *et al*[89] 2010 | Ireland | 47 | 43.6 (24–66) | 0 | - | - | - | 33 | Feces, Colonic mucosa | PCR-DGGE | Significantly more variation in the gut microbiota of healthy volunteers than that of IBS patients |
| Ponnusamy *et al*[90] 2011 | South Korea | 11 | 47.5 (18-74) | 6 | - | - | | 8 | Feces | DGGE + qPCR of *16S rRNA* genes | ↑ diversity of Bacteroidetes and Lactobacillus groups |
| Rinttilä *et al*[91] 2011 | Finland | 96 | 47 (20-73) | 27 | 15 | | 81 | 23 | Feces | qPCR | 17% of IBS samples ($n$ = 15) tested positive for staphylococcus aureus |
| Rajilić-Stojanović *et al*[45] 2011 | Finland | 62 | 49 (22−66) | 5 | 18 | 25 | 19 | 42 | Feces | Phylogenetic 16S rRNA microarray and qPCR | 2-fold ↑ firmicutes:Bacteroidetes ratio↓ bacteroidetes, ↑ dorea, ruminococcus, clostridium sppBifidobacterium faecalibacterium spp |
| Carroll *et al*[51] 2011 | United States | 16 | 35.6 (23–52) | 5 | 0 | 16 | 0 | 21 | Feces, Colonic biopsies | T-RFLP fingerprinting of 16S rRNA-PCR | ↓ microbial biodiversity in D-IBS fecal samples |
| Parkes *et al*[52] 2012 | United Kingdom | 53 | IBS-D: 36.2 (32.1–40.3), IBS-C: 32.4 (28.1–36.7) | 28 | 26 | 27 | 0 | 26 | Colonic mucosa | FISH, confocal microscopy | Expansion of mucosa-associated microbiota; mainly bacteroides and clostridia; association with IBS subgroups and symptoms |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Jeffery *et al*[92] 2012 | Sweden | 37 | 37 ± 12 | 11 | 10 | 15 | 12 | 20 | Feces | Pyrosequencing 16SrRNA | Clustering of IBS patients: normal-like *vs* abnormal microbiota composition (increase of firmicutes-associated taxa and a depletion of bacteroidetes-related taxa) |

IBS: Irritable bowel syndrome; IBS-D: Diarrhea-predominant irritable bowel syndrome; IBS-C: Constipation-dominant irritable bowel syndrome; IBS-M: Alternating type or mixed irritable bowel syndrome; PCR-DGGE: PCR denaturing gradient gel electrophoresis; FISH: Fluorescent *in situ* hybridization; qPCR: Quantitative PCR; 16S rRNA: 16S ribosomal RNA.

some of the outer mucus layers and, in turn, the mucosa-attached microbes as well as their normal attachment sites[16]. In addition, different studies used different sample handing methods; some studies used frozen samples, whereas others used fresh samples. The use of single samples cannot be linked to fluctuating symptoms and probably to other factors such as diet and patients' phenotypic characterization[7]. Although most studies used the Rome criteria for IBS, the proportions of the enrolled numbers of IBS subtypes differed among the studies. There is suggestive evidence of an association of intestinal microbiota in certain IBS subtypes. Kassinen *et al*[43] pooled fecal samples by an IBS subgroup diarrhea-predominant IBS (IBS-D), constipation-dominant irritable bowel syndrome (IBS-C), and IBS mixed type (IBS-M) and controls, extracted the bacterial DNA, and analyzed it using high-throughput 16S rRNA sequencing. Population analysis found significant differences between each IBS subgroup and controls[43].

It is difficult to determine whether alterations in microbiota are the primary events that lead to the development of IBS or merely the secondary effects of the syndrome. Despite these difficulties, previous studies found that the intestinal microbiota of some IBS patients was different from that of healthy controls, and there does appear to be a consistent theme of *Firmicutes* enrichment and reduced abundance of *Bacteroides*.

## PATHOGENIC ROLE OF INTESTINAL DYSBIOSIS IN IBS

Intestinal microbiota can be divided into two distinct ecosystems: luminal bacteria, which are either dispersed in liquid feces or bound to food particles, and mucosa-associated bacteria, which are bound to a mucus layer adjacent to the intestinal epithelium[16]. Although microbial trafficking will occur between the two ecosystems with a distinct microenvironment, each ecosystem has the potential to play a different role in IBS symptomatology (Figure 1). Luminal microbiota constitutes the majority of the gastrointestinal tract microbiota and plays a crucial role in gut homeostasis. In IBS, luminal microbiota may play a key role in bloating and flatulence through carbohydrate fermentation and gas production. Bacterial fermentation of undigested carbohydrate leads to short-chain fatty acid production, with gaseous byproducts such as carbon dioxide, hydrogen, and methane. The metabolites and toxins of luminal microbiota can modulate the host immune system[44]. Rajilić-Stojanović *et al*[45] prepared a phylogenetic 16S rRNA microarray and performed qPCR using fecal samples from 62 IBS patients and 46 healthy adults. Adult patients with IBS had a two-fold greater ratio of *Firmicutes* to *Bacteroidetes* than controls, resulting from an approximately one-and-a-half-fold increase in the numbers of *Dorea*, *Ruminococcus*, and *Clostridium spp*. In addition, they observed a two-fold decrease in the number of *Bacteroidetes* and a one-and-a-half-fold decrease in the numbers of *Bifidobacterium* and *Faecalibacterium spp*[45]. Furthermore, the instability and temporal variation in the intestinal microbiota of IBS subjects was addressed, and a trend was noted wherein some *Clostridium spp.* increased and *Eubacterium spp.* decreased in IBS patients[46].

Meanwhile, the mucosal microbiota, although fewer in number, may influence the host *via* immune-microbial interactions[35]. Recently, mucosal microbiota has attracted increased research interest. Mucosal microbiota is bound to a mucus layer consisting of glycosylated polysaccharides and glycocalyx. The mucus layer contains binding sites for commensal and pathogenic bacteria that help minimize adherence to the intestinal epithelium below. The vast majority of the microbiota is trapped in a complex biofilm containing a diverse population, and only those bacteria that are able to penetrate the mucus and that possess suitable adhesion proteins can directly interface with the apical surface[47]. Luminal interaction occurs *via* pattern recognition receptors such as toll-like receptors (TLRs) and NOD2. TLRs are expressed on the apical and basolateral membranes of enterocytes and on the processes of dendritic cells that pass from the lamina propria into the lumen through tight enterocyte junctions. Differential expression of TLRs was observed in patients with IBS, with increased TLR-4 and TLR-5 expression and decreased TLR-7 and TLR-8 expression compared with controls[48]. In addition, bacteria can pass through the epithelial layer and are presented to dendritic cells. The pathogenicity of the bacteria determines whether the dendritic cells either auto-induce tolerance *via* the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β or respond aggressively. Studies have also shown that bacteria such as *Bifidobacteria* and *Lactobacilli* stimulate IL-10 and TGF-β production by dendritic cells and inhibit the release of proinflammatory cytokines from dendritic cells[49]. A recent study revealed that some *Bifidobacterium* strains showed the highest production of IL-17 as well as poor secretion of interferon γ and tumor necrosis factor α, suggesting stimulation of the Th17 pathway[50]. The plasticity of

**Table 2 Advantages and limitations of the principal techniques used in the characterization of the intestinal microbiota[16,39]**

| | Advantages | Limitations |
|---|---|---|
| Culture | Cheap, easy to use | Limited estimate intestinal microbiota |
| PCR-T/DGGE | High sensitivity in detecting difference in bacterial populations, semi-quantitative | Does not identify bacteria unless bands on the gel are cut out and sequenced |
| FISH | Microbial *in situ* identification, high sensitivity, quantitative | Few species can be simultaneously detected, only known species are detected |
| T-RFLP | Low cost | Low biodiversity resolution, no species-level identification, not quantitative |
| Quantitative PCR | Can detect small number of bacteria and quantify them | Laborious |
| Phylogenetic microarray | High biodiversity resolution, quantitative | Only known species are detected |
| NGS phylogenetic analysis (*e.g.*, pyrosequencing) | Enormous quantities of data at individual Species level | Very costly, need bioinformatics analysis |

16S rRNA: 16S ribosomal RNA; PCR-T/DGGE: PCR temperature/denaturing gradient gel electrophoresis; FISH: Fluorescent *in situ* hybridization; T-RFLP: Terminal restriction fragment length polymorphism; qPCR: Quantitative PCR; NGS: Next-generation sequencing.

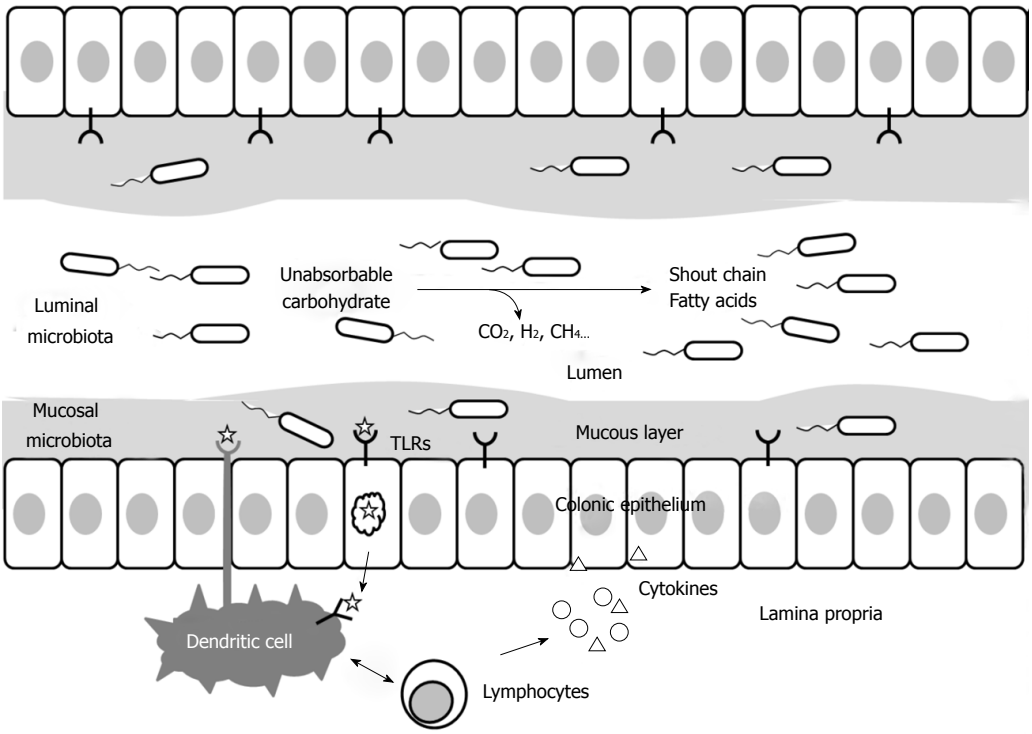


**Figure 1 Luminal and mucosal intestinal microbiota and roles in gut homeostasis.**

Treg/Th17 populations and the commensal bacteria play a key role in mucosal tolerance and T cell reprogramming[50]. It is, therefore, readily apparent that a disturbance in the mucosal microbiota could lead to an upregulation of the immune system. However, recent studies that examined the mucosal microbiota of IBS patients reported different results. Carroll *et al*[51] performed microbial community composition analyses on fecal and mucosal samples from patients with IBS-D and healthy controls using terminal-restriction fragment length polymorphism fingerprinting of the bacterial 16S rRNA gene. There were compositional differences in the luminal- and mucosal-associated microbiota of IBS-D patients and those of healthy controls as well as diminished microbial biodiversity in the IBS-D fecal samples. There were no differences in the biodiversities of the mucosal samples of IBS-D patients and healthy controls[51]. In contrast, Parkes *et al*[52] performed an analysis of frozen rectal biopsies taken at colonoscopy and bacterial quantification by hybridizing frozen sections with bacterial-group-specific oligonucleotide probes. They found expansion of mucosa-associated microbiota in IBS patients, mainly *Bacteroides* and *Clostridia*, and association with IBS subgroups and symptoms. In addition, they found that the mucosal *Bifidobacteria* were lower in IBS-D patients than in controls, together with a negative correlation between mucosal *Bifidobacteria* and the number of days patients experienced pain or discomfort. However, the studies on the mucosal microbiota of IBS patients are limited because doing so requires endoscopic examination of subjects' gastrointestinal tracts and carrying out biopsy, unlike the luminal microbiota, which can be readily examined in feces.

Intestinal microbiota may be involved in the pathogenesis of IBS by contributing to abnormal gastrointestinal motility, low-grade inflammation, visceral hypersensitivity, communication in the gut-brain axis, and so on. *Lactobacillus paracasei* NCC2461 significantly attenuated muscle dysfunction in a murine model of postinfective IBS[53]. The probiotic yeast *Saccharomyces boulardii* modulated the expression of neuronal markers in the submucous plexus of pigs[54]. There also seems to be an inflammatory component and dysregulation of pro- and anti-inflammatory cytokines in IBS patients[55]. Most interestingly, *Bifidobacterium infantis* (*B. infantis*) 35624 was shown to restore the balance of pro- and anti-inflammatory cytokines in patients[56]. *Lactobacillus farciminis* treatment prevented stress-induced hypersensitivity, increase in colonic paracellular permeability, and colonocyte myosin light chain phosphorylation in rats[57,58]. Modulation of the microbiota induces visceral hypersensitivity in mice, which is reduced by *L. paracasei* NCC 2461-secreted products[53]. Recently, Rousseaux *et al*[59] demonstrated that *Lactobacillus acidophilus* (*L. acidophilus*) contributes to the modulation and restoration of the normal perception of visceral pain through the NF-κB pathway and by inducing mu-opioid receptor 1 (MOR1) and cannabinoid receptor 2 (CB2) expression. Only the *L. acidophilus* NCFM strain was able to induce a significant *in vitro* expression of MOR1 and CB2 messenger in RNA and protein, respectively. To confirm these results *in vivo*, the researchers administered *L. acidophilus* NCFM orally to rats and mice at a clinically relevant concentration ($10^9$ CFU) and compared colonic samples from these rodents with those from untreated control rodents. MOR1 and CB2 expression was induced in 25%-60% of the intestinal epithelial cells from treated animals compared with only 0%-20% of those from the control group. In addition, visceral perception was assessed in rats using colorectal distension. Oral administration of the *L. acidophilus* NCFM strain for 15 d decreased normal visceral perception in the rats and increased their pain threshold by 20%. In further experiments of chronic colonic hypersensitivity on a rat model, treatment with *L. acidophilus* NCFM resulted in an analgesic effect similar to that of 1 mg morphine administered subcutaneously, thus increasing the colorectal distension threshold by 44% compared with that in untreated rats[59]. Transient perturbation of the microbiota with antimicrobials alters brain-derived neurotrophic factor expression, exploratory behavior, and colonization of germ-free mice, suggesting that the impact of the intestinal microbiota is not limited to the gut and the immune system[60].

## SMALL INTESTINAL BACTERIAL OVERGROWTH AND ANTIBIOTICS

Since Pimentel *et al*[61] reported that 84% of IBS patients had small intestinal bacterial overgrowth (SIBO) and that patients with IBS were over 26 times more likely to harbor SIBO than controls, the potential role of SIBO in IBS pathogenesis has gained considerable research attention[62]. In addition, bacterial fermentation in IBS has been highlighted in recent studies on SIBO[16]. Bacterial overgrowth in stagnant sections of the small intestine leads to malabsorption, diarrhea, bloating, and pain, and it can be treated with antibiotics. However, a subsequent study on the SIBO-IBS link showed similar results, whereas other studies were unable to establish an association[62].

A SIBO diagnosis test includes jejuna aspirate and culture, $^{14}$C-xylose breath test, and hydrogen ($H_2$) breath tests (HBT) using either glucose (GHBT) or lactulose (LHBT) as the substrate. Jejunal aspirate and culture is considered as the gold standard (> $10^5$ CFU after 48 h of culture); however, it is invasive and time consuming. In contrast, HBT is noninvasive and cheap, but prone to error. Following the ingestion of glucose or lactulose, serial breath $H_2$ measurements are performed. SIBO is defined by either a rise in $H_2$ > 20 ppm in < 90 min or a "double peak" demonstrating distinct small intestinal and colonic bacterial populations[63]. Meta-analysis of 12 studies containing 1921 subjects meeting the Rome criteria for IBS revealed that the pooled prevalence of a positive LHBT or GHBT was 54% (95%CI: 32%-76%) and 31% (95%CI: 14%-50%), respectively, but showed marked statistical heterogeneity between study results[64]. In addition, the prevalence of a positive jejunal aspirate and culture was only 4% (95%CI: 2%-9%). These results suggested that it is premature to accept a firm etiologic link between SIBO and IBS. Moreover, despite a decade of investigation on the relationship between SIBO and IBS, it remains unclear whether SIBO causes IBS or is a bystander of something else altogether[62].

However, the idea of treating IBS patients with an antibiotic was developed as a consequence of the SIBO concept[65]. Neomycin therapy eradicated SIBO and reduced symptoms of IBS[61,66]. Considering the chronic, relapsing nature of IBS and the undesirability of long-term systemic antibiotic therapy, the efficacy of rifaximin, a nonabsorbable antibiotic, began to be explored in IBS[67]. In a RCT, rifaximin treatment for 10 d resulted in symptom improvement that lasted for up to 10 wk in some IBS patients who did not document bacterial overgrowth[68]. Subsequently, a double-blind, placebo-controlled trial phase Ⅲ study reported that rifaximin treatment for 2 wk provided significant relief from IBS symptoms such as bloating, abdominal pain, and loose or watery stools[69]. A recent meta-analysis of 5 studies found rifaximin to be efficacious for global IBS symptom improvement (OR = 1.57, 95%CI: 1.22-2.01) and more likely to improve bloating (OR = 1.55, 95%CI: 1.23-1.96) compared with a placebo[70].

## EVIDENCE OF THE ROLE OF POTENTIALLY PROBIOTIC BACTERIA IN IBS

An improved understanding of host-microbiota interac-

**Table 3 Systemic reviews for randomized controlled trials of probiotics in irritable bowel syndrome**

| Ref. | Selection criteria | *n* of identified studies | Results |
|---|---|---|---|
| McFarland *et al*[73] 2008 | RCTs in humans published as full articles or meeting abstracts in peer-reviewed journals | 20 RCTs | Global IBS symptoms: RR = 0.77 (95%CI: 0.62-0.94)/ abdominal pain: RR = 0.78 (95%CI: 0.69-0.88) |
| Brenner *et al*[76] 2009 | RCTs; adults with IBS defined by Manning or Rome Ⅱ criteria; single or combination probiotic *vs* placebo; improvement in IBS symptoms and/ or decrease in frequency of adverse events reported | 16 RCTs → 11 studies showed suboptimal study design | *Bifidobacterium infantis* 35624 has shown efficacy for improvement of IBS symptoms. Most RCTs about the utility of probiotics in IBS have not used an appropriate study design |
| Hoveyda *et al*[74] 2009 | RCTs compared the effects of any probiotic therapy with placebo in patients with IBS | 14 RCTs → 7 RCTs providing outcomes as dichotomous variable and 6 RCTs providing outcomes as continuous variable | Overall symptoms: dichtomous data - OR = 0.63 (95%CI: 0.45-0.83)/continuous data - mean ± SD, 0.23 (95%CI: 0.07-0.38)<br>Trials varied in relation to the length of treatment (4-26 wk), dose, organisms and strengths of probiotics used |
| Moayyedi *et al*[75] 2010 | RCTs comparing the effect of probiotics with placebo or no treatment in adult patients with IBS (over the age of 16 yr) | 19 RCTs → 10 RCTs providing outcomes as a dichotomous variable | Probiotics appear to be efficacious in IBS (Probiotics were statistically significantly better than placebo, but there was statistically significant heterogeneity). The magnitude of benefit and the most effective species and strain are uncertain |
| Ortiz-Lucas *et al*[77] 2013 | RCTs comparing probiotics with placebo in treating IBS symptoms | 24 RCTs → 10 RCTs providing continuous data performed with continuous data summarized using mean ± SD and 95%CIs | Pain scores: improved by probiotics containing *Bifidobacterium breve*, *Bifidobacterium longum*, or *Lactobacillus acidophilus* species<br>Distension scores: improved by probiotics containing *B. breve*, *B. infantis*, *Lactobacillus casei*, or *Lactobacillus plantarum* species<br>Flatulence: improved by probiotics containing *B. breve*, *B. infantis*, *L. casei*, *L. plantarum*, *B. longum*, *L. acidophilus*, *Lactobacillus bulgaricus*, and *Streptococcus salivarius ssp. thermophilus* |

IBS: Irritable bowel syndrome; RCT: Randomized controlled trial.

tions in IBS is not only important for its pathogenesis but also for assessing the possible benefits of potential probiotic strains in IBS management. Probiotics are defined as live organisms that when ingested in adequate amounts yield a health benefit to the host[9]. Clinically acceptable probiotics should be species-specific; should be of human origin; should survive passage from the oral cavity through the gastric acid barrier, digestive enzymes, and bile acids; should travel down the small bowel into the colon; nidate; and should proliferate therein[54]. Probiotics offer protection against potential pathogens through enhancement of mucosal barrier function by secreting mucins; providing colonization resistance; producing bacteriocins; increasing production of secretory immunoglobulin A; producing a balanced T-helper cell response; and increasing production of IL-10 and TGF-β, both of which play a role in the development of immunologic tolerance to antigens. For example, a specific strain of *B. infantis* 35624 has been shown to prevent NF-κB and IL-8 activation as well as to inhibit the secretion of chemokine ligand 20 in response to *Salmonella typhimurium*, *Clostridium difficile*, and *Mycobacterium paratuberculosis*[71]. Current evidence suggests that probiotic effects are strain-specific[72].

Probiotics should be administered at an adequate dose, preferably greater than 10 billion CFU/g in adults; their viability and concentration should be maintained; and they should have a dependably measurable shelf life at the time of purchase and administration. When these criteria are fulfilled, randomized, placebo-controlled, double-blind trials should be performed on an appropriate population. Five systematic reviews with RCTs of adult IBS patients were published[73-77]. Most of the meta-analyses indicated a beneficial effect of probiotics on global symptoms, abdominal pain, and flatulence, whereas the influence on bloating was equivocal (Table 3). However, aggregation of the effects of different probiotics into a meta-analysis should be undertaken with caution. Different probiotics have different microbiological characteristics, which inevitably influence their efficacy. The most commonly studied probiotic species are *Lactobacilli* and *Bifidobacteria*. Products range in delivery systems (*e.g.,* yogurts, fermented milk drinks, powders, and capsules) and dose ($10^6$-$10^{10}$ CFU). *Lactobacillus plantarum*, *B. infantis*, and VSL 3 (*Lactobacillus casei*, *L. plantarum*, *L. acidophilus*, *Lactobacillus delbrueckii*, *Bifidobacterium longum*, *Bifidobacterium breve*, *B. infantis*, and *Streptococcus salivarius*) have demonstrated efficacy in patients with IBS[56,78,79].

Recently, we isolated have been isolated new strains, *i.e.*, *L. acidophilus*-SDC 2012, 2013, from Korean infants'

feces[8]. In Korea, the prevalence of IBS is reported to be around 2.2%-6.6%[1], while that in Western countries is around 10%-20%[2]. Based on the relatively lower prevalence of IBS in Korea and previous reports on the efficacy of probiotics for treating IBS symptoms, we hypothesized that the newly isolated *L. acidophilus*-SDC 2012, 2013 may help control the symptoms of IBS patients. The result of our RCT showed that *L. acidophilus*-SDC 2012, 2013 were effective in alleviating IBS symptoms, irrespective of the bowel habit subtype[8]. Although *Lactobacilli* or *Bifidobacteria* have demonstrated efficacy in IBS patients, the benefits of one given species or organism have not been found to be better than that of other species or organisms. In an RCT of composite probiotics, Kim *et al*[80] reported that VSL3 reduced flatulence and retarded colonic transit without altering bowel function in patients with IBS and bloating.

Recent guidelines published by the British Dietetic Association have therefore made strain-specific recommendations considering the limited weak evidence for *B. lactis* DN 173010 in improving overall symptoms, abdominal pain, and urgency in constipation-predominant IBS and the limited weak evidence for VSL3 in reducingg flatulence in IBS patients[32]. People with IBS who choose to try probiotics should be advised to consume a given product for at least 4 wk while monitoring the effect. Probiotics should be consumed at the dose recommended by the manufacturer[75,76,81].

A number of RCTs have been performed for investigating the effectiveness of probiotics in IBS. However, most RCTs of probiotics had a suboptimal study design with inadequate blinding, trial length, sample size, and/or lack of intention-to-treat analysis. Despite these limitations, there is a possibility of greater efficacy of probiotics in patients whose IBS pathogenesis is known to be related to the intestinal microbiota. In addition, the probiotics include strains present in normal intestinal microbiota, and probiotic-associated adverse events are very rare. Thus, probiotics are good candidates for controlling the symptoms of IBS, especially when treatment safety is paramount in a nonlethal disorder such as IBS[82]. The evidence from clinical trials and systematic reviews are largely supportive of the use of specific probiotics strains in IBS[9].

## CONCLUSION

Multiple recent studies have consistently proven that intestinal dysbiosis is associated with this IBS. An improved understanding of host-microbiota interactions in IBS is important not only for determining its pathogenesis but also for enabling therapeutic modulation of the microbiota. In addition, such evidence has encouraged investigations of the potential roles of antibiotics and probiotics in this disorder. Although the interactions of microbiota-targeted treatments with the host immune and visceral nervous systems are yet to be fully understood, they have the potential to play a key role in the management of IBS.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

# Irritable bowel syndrome and small intestinal bacterial overgrowth: Meaningful association or unnecessary hype

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## Abstract

Irritable bowel syndrome (IBS) is a common condition characterized by abdominal pain or discomfort, bloating, and altered stool form and passage. Small intestinal bacterial overgrowth (SIBO) is a condition in which there is overgrowth of bacteria in small bowel in excess of $10^5$ colony forming units per milliliter on culture of the upper gut aspirate. Frequency of SIBO varied from 4%-78% among patients with IBS and from 1%-40% among controls. Higher frequency in some studies might be due to fallacious criteria [post-lactulose breath-hydrogen rise 20 PPM above basal within 90 min (early-peak)]. Glucose hydrogen breath test (GHBT) has a low sensitivity to diagnose SIBO. Hence, studies based on GHBT might have under-estimated frequency of SIBO. Therefore, it is important to analyze these studies carefully to evaluate whether the reported association between IBS and SIBO is over or under-projected. This review evaluates studies on association between SIBO and IBS, discordance between different studies, their strength and weakness including methodological issues and evidence on therapeutic manipulation of gut flora on symptoms of IBS.



**Key words:** Glucose hydrogen breath test; Lactulose hydrogen breath test; Functional bowel disease; Dysbiosis; Gut flora

**Core tip:** Irritable bowel syndrome (IBS) has been conventionally thought to be a disorder without an organic basis. However, recently data are emerging to show that it may have organic basis at least in a subset of patients. Though several studies reported an association between small intestinal bacterial overgrowth (SIBO) and IBS, the frequency of SIBO reported to vary between 4% and 78%. The current review suggests that the association between SIBO and IBS is definite, but the studies reporting high prevalence of SIBO in IBS over-estimated its frequency due to use of fallacious diagnostic methods. Better test to diagnose SIBO in patients with IBS is highly needed.

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## INTRODUCTION

Irritable bowel syndrome (IBS) is a common condition characterized by abdominal pain or discomfort, bloating, associated with altered stool form (such as diarrhea, constipation) and passage. World-wide, 4%-30% of subjects suffer from IBS[1-7]. Small intestinal bacterial overgrowth (SIBO), which has been conventionally described in patients with anatomical abnormalities in the gut such

**Table 1 Summary of prevalence of small intestinal bacterial overgrowth in irritable bowel syndrome by different diagnostic methods *n* (%)**

| Ref. | Type of the study | Frequency of SIBO in cases | Frequency of SIBO in controls | Methane producers in cases | Methane producers in controls | Method for diagnosis |
|---|---|---|---|---|---|---|
| Park *et al*[86] | Prospective (Case-control) | 34/76 (44.7) | 16/40 (40) | 19/76 (25) | 10/40 (25) | LHBT |
| Scarpellini *et al*[88] | Prospective (Case-control) | 28/43 (65) | 4/56 (7) | 4/43 (9.3) | 0 | LHBT |
| Carrara *et al*[14] | Prospective | 55/127 (43) | NCG | ND | ND | LHBT |
| Mann and Limoges-Gonzales[87] | Prospective | 89/258 (34.5) | NCG | ND | ND | LHBT |
| Nucera *et al*[89] | Prospective | 64/98 (65) | NCG | ND | ND | LHBT |
| Pimentel *et al*[17] | Prospective | 157/202 (78) | NCG | ND | ND | LHBT |
| Sachdeva *et al*[18] | Prospective (Case-control) | 14/59 (23.7) | 1/37 (2.7) | 5/59 (8.5) | 9/37 (24.3) | GHBT |
| Reddymasu *et al*[90] | Prospective | 35/98 (36) | NCG | Data NA | ND | GHBT |
| Lombardo *et al*[91] | Prospective (Case-control) | 49/200 (24.5) | 3/50 (6) | ND | ND | GHBT |
| Ford *et al*[74] | Meta-analysis | 595/1921 (31) | NCG | ND | ND | GHBT |
| Parodi *et al*[92] | Prospective (Case-control) | 21/130 (16.1) | 3/70 (4.2) | 34/130 (26) | Data NA | GHBT |
| Rana *et al*[93] | Prospective (Case-control) | 25/225 (11.1) | 1/100 (1) | ND | ND | GHBT |
| Majewski *et al*[94] | Prospective (Case-control) | 93/204 (46) | NCG | 27/204 (13.2) | ND | GHBT |
| Cuoco and Salvangnini[75] | Retrospective | 44/96 (45.8) | NCG | ND | ND | GHBT |
| Lupascu *et al*[95] | Prospective (Case-control) | 20/65 (31) | 4/102 (4) | ND | ND | GHBT |
| Ghoshal *et al*[16] | Prospective (Case-control) | 11/129 (8.5) | 1/51(2) | ND | ND | GHBT |
| Posserud *et al*[12] | Prospective (Case-control) | 6/162 (4) | 1/26 (4) | ND | ND | Hydrogen breath test and culture of small bowel aspirate |

SIBO: Small intestinal bacterial overgrowth; IBS: Irritable bowel syndrome; LHBT: Lactulose hydrogen breath test; GHBT: Glucose hydrogen breath test; NCG: No control group; ND: Not done; NA: Not available; *n*: Number of cases and controls.

as ileo-transverse anastomosis, stricture, fistula, slow motility and reduced gut defence, is also characterized by abdominal pain or discomfort, bloating, flatulence and loose motion[8-10]. Recently, it has been realized that SIBO may occur in the absence of apparent anatomical abnormalities[11]. These patients, however, may be wrongly diagnosed as IBS.

Small intestinal bacterial overgrowth is currently defined as presence of bacteria in excess of $10^5$ colony forming units per milliliter on culture of the upper gut aspirate[12,13]. Since this is an invasive test, several non-invasive methods including lactulose and glucose hydrogen breath tests (LHBT and GHBT) have been popularly used to diagnose SIBO[14-18]. This condition is being increasingly recognized among patients with IBS. In different studies, frequency of SIBO among patients presenting with IBS varied from 4% to 78% (Table 1), more so among patients with diarrhea-predominant IBS[12,14,18,19]. Not only quantitative increase (SIBO) but qualitative change in the gut bacteria (dysbiosis) has been reported among patients with IBS[20]. These studies led to a paradigm shift in understanding pathogenesis of IBS and have led to increasing debate on therapeutic manipulation of gut microbiota to treat this enigmatic chronic disorder using antibiotics, probiotics and lately fecal transplantation[21-23]. However, it is important to recognize the wide-variability in frequency of SIBO among patients with IBS in different studies; such wide-variability in frequency may suggest that it is important to analyze these studies carefully to evaluate whether the reported association between IBS and SIBO is over or under-projected in some of the earlier studies?

We hereby review the studies on association between SIBO and IBS, discordance between different studies, their strength and weakness including methodological issues and evidence on therapeutic manipulation of gut flora on symptoms of IBS.

## REVIEW OF STUDIES ON SIBO IN IBS

Table 1 summarizes the results from studies on SIBO among patients with IBS. As can be noted from this table, the frequency of SIBO among patients with IBS varied from 4% to 78% and that among controls from 1% and 40%. In most studies, frequency of SIBO among patients with IBS was higher than that among controls. Therefore, it can be concluded that SIBO is associated with IBS. But it is important to critically evaluate the reasons for such a wide variability in frequency of SIBO in different studies.

## CRITICAL EVALUATION OF STUDIES ON SIBO IN IBS: REASONS FOR DISCORDANCE

### *Could IBS phenotype determine frequency of SIBO?*

IBS is a heterogeneous condition. The sub-types may be diarrhea or constipation-predominant or may be alternating. Patients with diarrhea-predominant IBS more often have organic cause including SIBO than other sub-types of IBS. In a study on 129 patients with non-diarrheal IBS, 73 with chronic non-specific diarrhea including diarrhea-predominant IBS, and 51 healthy controls, frequency of SIBO using GHBT was 11 (8.5%), 16 (22%) and 1 (2%), respectively[16]. Similar findings were reported in other studies as well[18,24]. Diarrheal IBS, therefore, should be particularly evaluated for SIBO than other types of IBS. Moreover, studies which included larger proportion of patients with diarrhea-predominant

IBS are likely to show higher frequency of SIBO.

Bloating is a frequent symptom reported by patients with IBS. Frequency of bloating has been reported to vary from 26% to 83% in studies on IBS from Asia[3,25]. The pathogenesis of bloating may be related to increased amount of gas in the gut, its abnormal distribution and increased visceral perception in response to distension of the gut[26,27]. Patients with SIBO may have increased amount of gas inside the gut, hence, it is logical to believe that IBS patients with marked bloating are expected to have SIBO[16]. However, there are limited data on this issue. Several studies showed that both fasting as well as post-substrate (*e.g.*, glucose, lactulose) breath hydrogen is higher among patients with IBS than controls[16,28]. Probiotics and antibiotics, which are known to reduce gas inside the gut, are known to relieve abdominal bloating among patients with IBS[17,29,30]. It has been reported that successful treatment with antibiotics causes the hydrogen breath test to revert to normal[17]. Hence, patients with IBS with marked bloating and flatulence should be particularly evaluated for SIBO. More studies, however, are needed on this issue.

### *Could technique used to diagnose SIBO determine its frequency?*

Several techniques have been used to diagnose SIBO; these include LHBT, GHBT, $^{14}$C xylose breath test, and quantitative culture of jejunal aspirate[12,15,31,32]. Principle of hydrogen breath tests is summarized in Figure 1. Dietary carbohydrates, unabsorbed in the small intestine, produce hydrogen in the large intestine by bacterial fermentation[33]. In patients with SIBO, the bacteria ferment these carbohydrates in the small bowel itself producing hydrogen, which gets absorbed and is exhaled in the breath[33].

Hydrogen breath test involves giving patients a load of carbohydrate (usually in the form of glucose and lactulose) and measuring expired hydrogen concentrations over a period of time. Diagnosis of SIBO using hydrogen breath test is based on the physiological principle that in patients with SIBO, glucose would be fermented by bacteria in the small bowel resulting in production of hydrogen gas that is absorbed and exhaled in expired air (Figure 1, A1)[31,33]. In contrast, lactulose, which is a non-absorbable disaccharide, will produce an early peak due to fermentation in small bowel (typically within 90-min) or double peak (first due to small bowel fermentation and second from colon), if SIBO is present (Figure 1, B2 and B3)[33]. There are several limitations in hydrogen breath test for diagnosis of SIBO. There may be similarities in the pattern of gas production in patients with SIBO and subjects with rapid intestinal transit, thus making distinction difficult[34]. An early peak is often false positive in people with fast gut transit time. For example, in a study from India, median oro-cecal transit time was 65 min (range 40-110 min) in healthy subjects[35]. In another study from Taiwan, mean oro-cecal transit time was 85 min[36]. This has also been substantiated in Western population recently by simultaneously using LHBT and radio-nuclide method to assess gut transit[37-39]. Double peak criteria for diagnosis of SIBO using LHBT is quite insensitive[15,33]. Sensitivity of GHBT to diagnose SIBO is 44% considering quantitative culture of upper gut aspirate as gold standard[15]. Hence, it is expected that the authors who used an early peak criteria in LHBT would get a high frequency of SIBO among patients with IBS as well as controls. In contrast, those who would use either GHBT or double peak criterion in LHBT would get a low frequency of SIBO both in patients with IBS and controls. It is worth noting from the Table 1 that the frequency of SIBO among patients with IBS and controls on LHBT (early peak criteria) varied from 34.5% to 78% and 7%-40%, respectively; in contrast, the frequency on GHBT varied from 8.5%-46% and 2%-18%, respectively.

Fifteen percent of people may have methanogenic flora in the gut[34,40]. *Methanobrevibacter smithii*, *Methanobrevibacter stadmanae* and possibly some of the coliform bacteria are methanogens[41]. In these subjects, only hydrogen breath tests may not diagnose SIBO, estimation of methane is also needed (Figure 1). Table 1 shows that 8.5%-26% of IBS patients and 0%-25% of controls exhaled methane in their breath. Therefore, SIBO could not have been diagnosed if methane was not estimated in them. As summarized in Table 1, in a large proportion of studies, methane was not estimated, which could have resulted in underestimation of frequency of SIBO in these studies. Excessive methane production is associated with constipation[42]. Hence, methane estimation in breath, which is not available in several commercially available hydrogen breath test machines, is particularly important in patients with constipation-predominant IBS. Some individuals may have slow transit through the small intestine making prolonged testing up to five hours necessary and many individuals may not like to undergo such prolonged testing[43,44]. However, a shorter period of testing for them may miss the diagnosis of SIBO.

The jejunal aspirate culture has traditionally been used as the gold standard to diagnose SIBO (Figure 2)[15,45]. However, the limitations of this test include invasiveness and the challenges posed by attempting to culture all strains and species[46]. In fact, use of air during endoscopy can lead to a false negative result as anaerobes do not grow once these are exposed to oxygen present in the air[13]. Also, a large proportion of bacteria are not cultured[47,48]. In contrast, single lumen catheter passed through the nose or through the biopsy channel of endoscope, may lead to contamination by oropharyngeal flora giving false positive result[13]. Therefore, we designed a double-lumen catheter to prevent such oro-pharyngeal contamination (Figure 2)[13]. Studies on SIBO among patients with IBS using quantitative culture of small bowel aspirate are scanty (Table 1). A study by Posserud *et al*[12] reported a frequency of SIBO of 4% among patients with IBS. Considering the result of other studies using GHBT, which has sensitivity of 44%

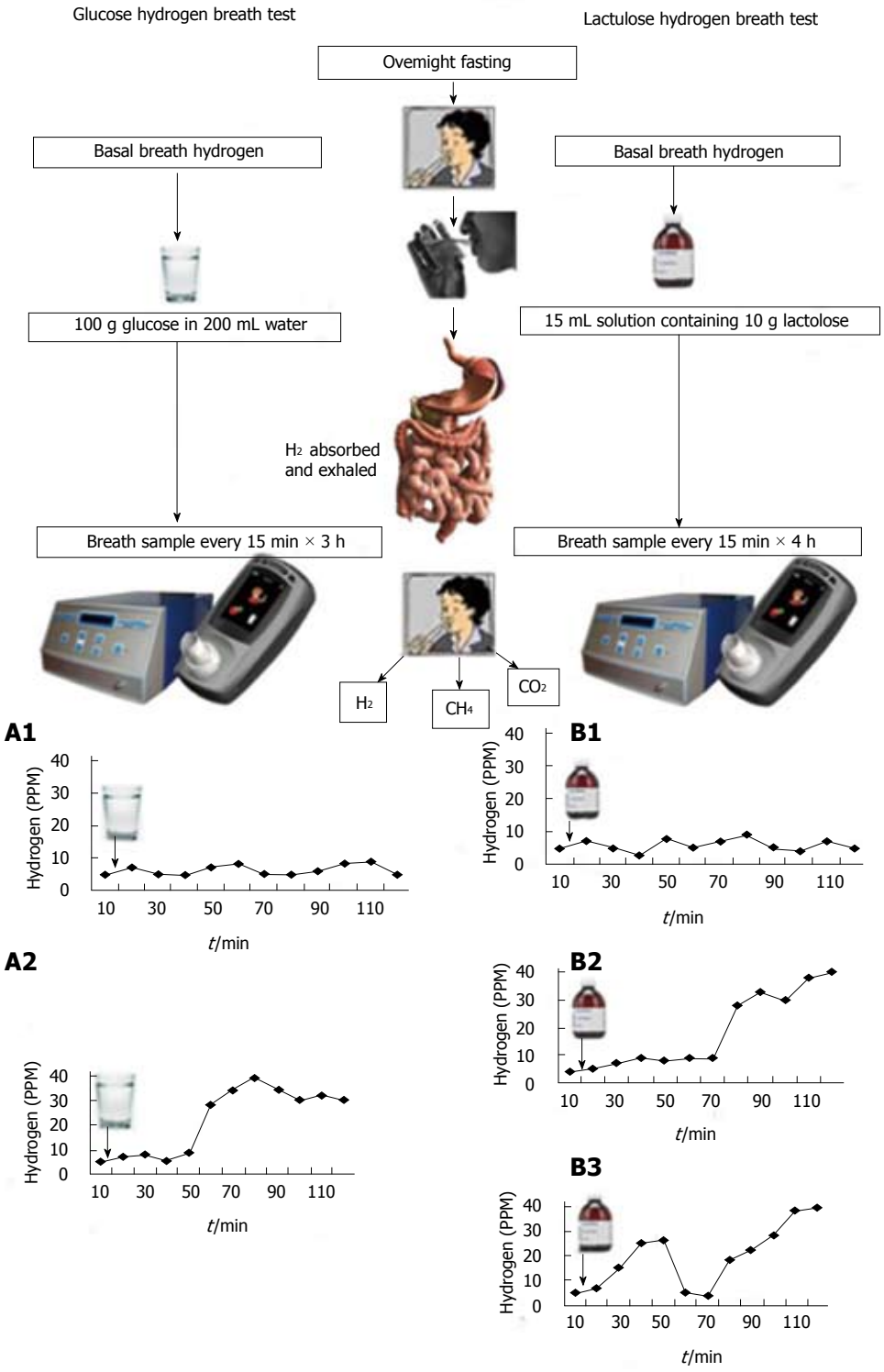


**Figure 1 Outline of principle of method and interpretation of glucose and lactulose hydrogen breath tests.** On the left panel, method and result of glucose hydrogen breath test (GHBT) is shown. A1: GHBT is negative for SIBO as there is no peak in hydrogen production; A2: GHBT shows presence of SIBO. On the right panel, method and result of lactulose hydrogen breath test (LHBT) is shown. B1: LHBT is negative for SIBO as there is no peak in hydrogen production; B2: LHBT shows an early peak (within 90 min of lactulose ingestion); B3: LHBT shows double peaks in hydrogen, the earlier one from small bowel due to SIBO and the later one from the colon. Please note that Quintron machine of the left gives values of hydrogen, methane and $CO_2$ (for correction) and the Bedfont machine of right side estimates hydrogen only. It is also important to note that in the graphs hydrogen breath test do not show methane levels. SIBO: Small intestinal bacterial overgrowth; GHBT: Glucose hydrogen breath test; LHBT: Lactulose hydrogen breath Test; PPM: Parts per million.

to diagnose SIBO considering upper gut aspirate as gold standard, the former study appears to have falsely underestimated the frequency of SIBO among patients with IBS[15]. More studies are needed on this issue.

$^{13}C$ and $^{14}C$ based tests have also been developed based on the bacterial metabolism of D-xylose (Figure

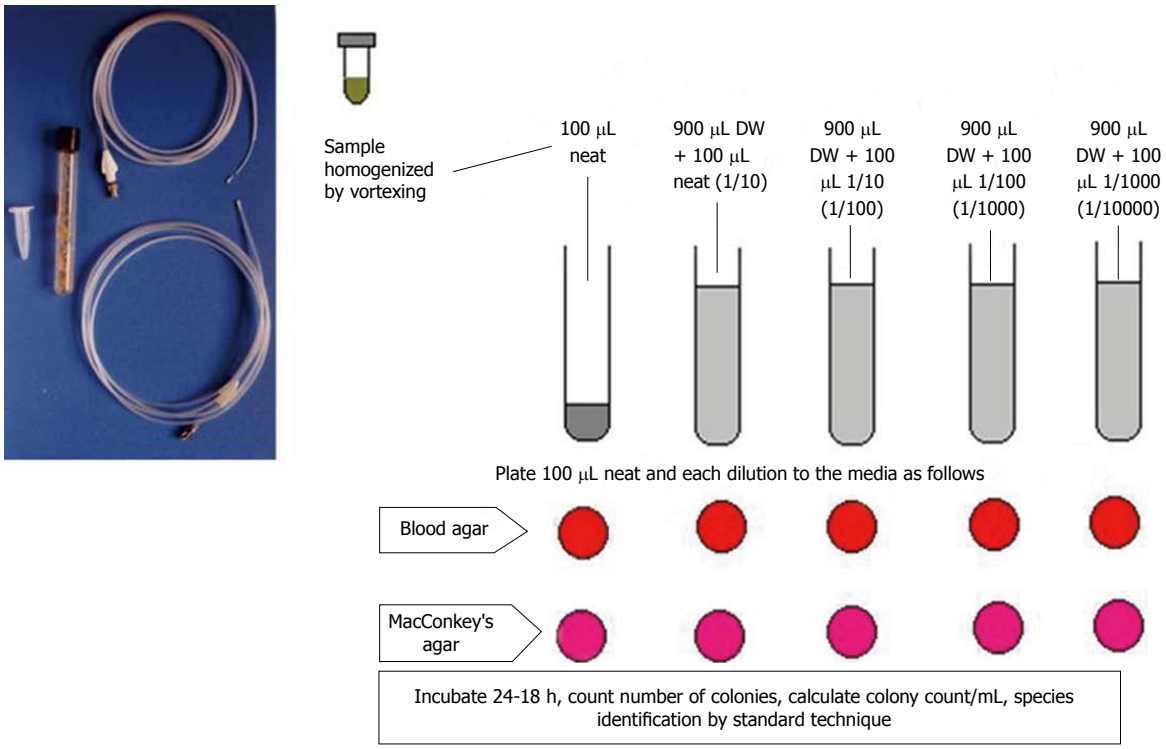


**Figure 2 Outline of method of culturing bacteria from the upper gut aspirate and counting the colonies by serial dilution technique.** DW: Distilled water.

3)[32]. Bacterial de-conjugation of bile acids containing $^{13}C$ and $^{14}C$ can also be used to diagnose SIBO[49]. The glycocholic acid breath test involves the administration of the bile acid $^{14}C$ glycocholic acid, and the detection of $^{14}CO_2$, which would be elevated in SIBO (Figure 3)[50,51].

## MECHANISM OF IBS SYMPTOMS IN PATIENTS WITH SIBO

Bacteria in gut lumen play important role in modulating multiple intestinal functions. Quantitative change in luminal bacteria in the small intestine (SIBO) disrupts digestion and absorption. Gut bacteria are also important for immune activation[52]. Immune mediated cytokines could have multiple actions including altered epithelial secretion, exaggerated nociceptive signalling and abnormal motility[52,53]. Together, these changes may lead to IBS like symptoms. It has also been proposed that this mechanism could account for overlap syndromes, such as fibromyalgia[54,55]. There has been renewed interest in gut flora recently, as there are recent developments on relationship between gut flora and intestinal function, pathogenesis of various diseases and potential value of probiotics, and other means of modifying gut flora as therapeutic modalities.

In patients with SIBO, bacteria ferment ingested carbohydrates in the small intestine causing increased gas production[15]. Accumulation of this gas in the intestine results in bloating and flatulence[56,57]. Excessive luminal distension may even cause abdominal pain or discomfort[57]. 15% of the population produces methane instead of hydrogen gas[13,34]. Evidence suggest that excessive methane produced by overgrowth of methanogenic flora causes constipation[42]. Reduction in breath methane by therapeutic manipulation of gut flora improves constipation[58].

Bacteria in the intestine may produce toxic by-products after fermentation, which may damage the inner lining of the small intestine and colon[59]. A study on small bowel biopsies in patients with SIBO revealed thinning of the mucosa and crypts and increased intra-epithelial lymphocytes[60]. This may cause osmotic load in the intestine resulting into diarrhea. Studies have revealed that patients with IBS having SIBO more often have diarrhea-predominant disease[11,12,14,18,19]. Bacteria also de-conjugates bile salts present in the intestine. These de-conjugated bile salts can stimulate colonic water secretion causing diarrhea. Thereafter, free bile acids, which are toxic to the intestinal mucosa may cause mucosal inflammation and release of pro-inflammatory cytokines[61,62]. SIBO is known to be associated with increased IL-8 levels (pro-inflammatory cytokine)[63].

Pathophysiology of IBS includes altered motility, visceral hypersensitivity and abnormal brain-gut interaction. Bacteria affect the sensori-motor functions of the gut[64]. Bacterial chemotactic peptides, such as formyl-methionyl-leucyl-phenylalanine, stimulate the enteric nervous system and afferent nerves, while endotoxins (lipopolysaccharides) may affect gut motility[65]. Bacteria in the small intestine in patients with SIBO produce short-chain fatty acids (SCFA) such as butyrate, acetate, and propionate. Colonic motility is increased due to acidification by SCFA[66,67]. In contrast, SCFA causes reduction in motil-

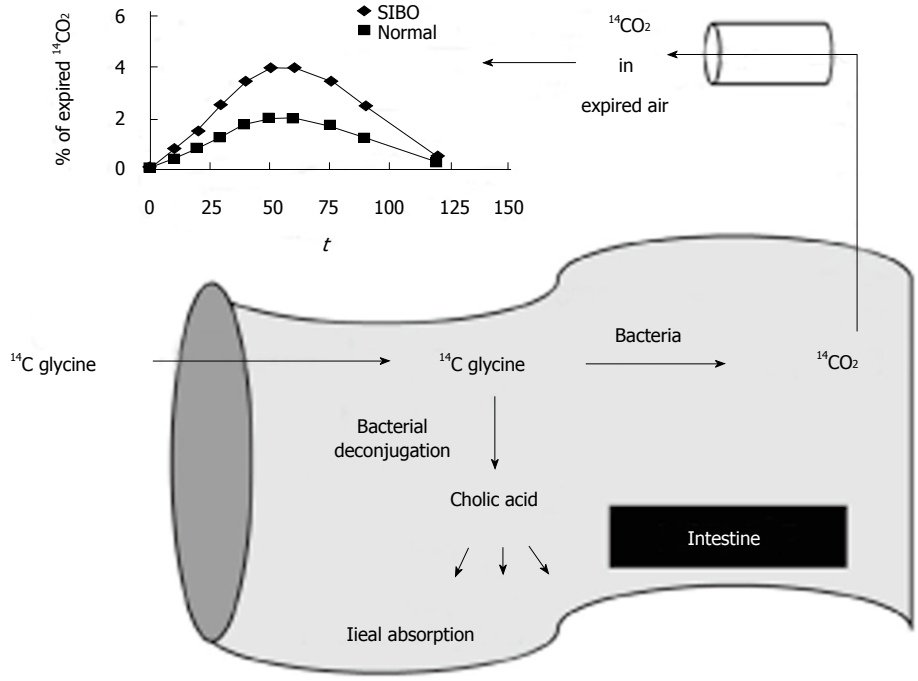


**Figure 3 Bile acid breath test involves the administration of the bile acid $^{14}C$ glycocholic acid, and the detection of $^{14}CO_2$, which would be elevated in small intestinal bacterial overgrowth.**

ity of proximal intestine due to release of peptide YY, neurotensin and glucagon-like peptide-1 in the ileum[68]. Therefore, alteration in gut flora may cause altered motility and predispose to IBS like symptoms.

## EVIDENCE FROM STUDIES ON THERAPEUTIC MANIPULATION OF GUT FLORA IN IBS

Gut flora of IBS patients is different from that of healthy subjects, resulting in more gas production due to bacterial fermentation[69-71]. Evidence suggest that therapeutic manipulation of gut flora either with antibiotics or probiotics may lead to relief in symptoms of IBS[72,73]. Several meta-analysis have reported the presence of SIBO in a subset of IBS patients[14,17,74]. Recent studies have revealed that treatment of SIBO relieves symptoms of IBS[17,75]. In a study, eradication of SIBO by open label antibiotic treatment resolved symptoms of IBS to the extent of Rome I criteria turning negative in 48% of patients[17]. SIBO is more often found in diarrheal IBS than other subtypes[16]. Treatment of patients with diarrhea-predominant IBS with antibiotics, which is the primary treatment of SIBO, may lead to relief in symptoms including bloating, abdominal pain and loose stools. Rifaximin, a non-absorbable broad spectrum antibiotic, has been widely used for treatment of SIBO[76]. In a phase 3, double-blind, placebo-controlled trial on IBS patients without constipation, treatment with rifaximin provided significant relief in bloating, abdominal pain, and loose or watery stools as compared to placebo[77].

Methane produced by methanogenic flora is shown to cause slow transit constipation, which is associated with IBS[78]. Recently, rifaximin is found to reduce methane gas and improve gut transit[58]. A combination of rifaximin and neomycin is more effective in treating methane-producing IBS patients as compared to treatment with neomycin and rifaximin as single agents (87% *vs* 33% and 28%, respectively)[79].

Some studies support the use of probiotics to be as effective as antibiotics in relieving IBS related symptoms[80,81]. A study showed that treatment with probiotics was effective in reducing symptoms of abdominal pain, bowel frequency, urgency and distension in patients with chronic diarrhea[82]. Lactobacilli are less gas producing than other bacteria[70]. Therefore, administration of Lactobacilli in patients with IBS was associated with reduced gas-related symptoms[83]. In a single blinded randomized control trial, IBS patients randomized to receive *Lactobacillus acidophilus*, *Lactobacillus helveticus*, and Bifidobacterium showed significant improvement in pain and bloating as compared to those who received placebo[84]. Another study showed *Bacillus subtilis* and *Streptococcus faecium* to be effective in reducing abdominal pain as compared to placebo in patients with diarrhea or alternating type of IBS[85]. However, more studies are needed to know the best strains of probiotic bacteria, their dose and duration for treatment of patient with IBS.

## CONCLUSION

Association between IBS and SIBO is definite. In fact, controversy exists whether patients presenting with IBS but found to have SIBO on further testing should be diagnosed as IBS or should be considered as SIBO as

symptoms of IBS and SIBO are similar. However, in the current diagnostic algorithm of Rome Foundation, IBS is diagnosed by symptom-based criteria. Exclusion of SIBO by appropriate testing is not essential before diagnosing IBS. In future iteration on IBS diagnostic algorithm by Rome Foundation, it may be important that consideration is given to exclude SIBO before a diagnosis of IBS is made, at least in a subset of patients with higher probability of SIBO. However, as evident from the review of the existing data, we conclude that whereas in some studies, the frequency of SIBO was over-estimated, in others it was under-estimated. Studies that used an early-peak criteria on LHBT showed higher frequency of SIBO than those that used other methods to diagnose. In the context of recent studies that showed that early-peak criteria on LHBT if often false positive, it is likely that all these studies over-estimated frequency of SIBO and therefore, created an un-necessary hype[14,17,86,87]. GHBT has a low sensitivity to diagnose SIBO[15]. Therefore, studies based on GHBT might have under-estimated frequency of SIBO. Though jejunal aspirate culture is considered as gold standard for diagnosis of SIBO, this has limitations. Most importantly, this is invasive and hence, not acceptable to the patients. Therefore, there is urgent need to know the clinical predictors for considering diagnosis of SIBO in patients presenting as IBS and better diagnostic techniques to confirm this.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

# Melatonin for the treatment of irritable bowel syndrome

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## Abstract

Irritable bowel syndrome (IBS) is a common disorder characterized by recurrent abdominal pain or discomfort, in combination with disturbed bowel habits in the absence of identifiable organic cause. Melatonin (N-acetyl-5-methoxytryptamine) is a hormone produced by the pineal gland and also large number by enterochromaffin cells of the digestive mucosa. Melatonin plays an important part in gastrointestinal physiology which includes regulation of gastrointestinal motility, local anti-inflammatory reaction as well as moderation of visceral sensation. Melatonin is commonly given orally. It is categorized by the United States Food and Drug Administration as a dietary supplement. Melatonin treatment has an extremely wide margin of safety though it may cause minor adverse effects, such as headache, rash and nightmares. Melatonin was touted as a potential effective candidate for IBS treatment. Putative role of melatonin in IBS treatment include analgesic effects, regulator of gastrointestinal motility and sensation to sleep promoter. Placebo-controlled studies in melatonin suffered from heterogeneity in methodology. Most studies utilized 3 mg at bedtime as the standard dose of trial. However, all studies had consistently showed improvement in abdominal pain, some showed improvement in quality of life of IBS patients. Melatonin is a relatively safe drug that possesses potential in treating IBS. Future studies should focus on melatonin effect on gut mobility as well as its central nervous system effect to elucidate its role in IBS patients.



**Key words:** Melatonin; Irritable bowel syndrome; Pain; Sleep; Analgesia

**Core tip:** Irritable bowel syndrome (IBS) is a common disorder associated with significant disability and high social cost. This is partly due to lack of effective treatment with low side effects. Melatonin is a drug that was postulated to be a potential useful arsenal in battling IBS. Its role in analgesia has been recognized in several other fields of medicine. Several well-designed placebo-controlled trials in IBS patients had consistently showed improvement of abdominal pain when taking 3 mg of melatonin with no serious side effect. Future studies should examine the long term effect of Melatonin as well as its effect on central nervous system and gut motility.

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## INTRODUCTION

Irritable bowel syndrome (IBS) is a common disorder characterized by recurrent abdominal pain or discomfort, in combination with disturbed bowel habits in the absence of identifiable organic cause. It is associated with significant disability and health care costs. In the West,

the prevalence of IBS in the community is reported to be between 10%-20%[1-3]. In Asia, we have also seen a steady rise of IBS in the community. In Singapore, prevalence of IBS was reported to be 2.3%[4] in 1998, by the Manning criteria, and 8.6% in 2004 (as defined by the Rome Ⅱ criteria)[5]. In addition, a recent study has shown that the disease burden extends beyond the patient and has significant impact on the spouse or family members as well, with burden proportionally increasing with IBS severity[6], underscoring the need for effective treatment of the patient's symptoms.

Traditionally, IBS is treated with a combination of treatment modality, from antispasmodic, psychopharmacological treatment like tricyclic antidepressant to mindfulness therapy like hypnotherapy. Newer drugs such as linaclotide, prucalopride, tegaserod and lubiprostone[7] have given hope to clinicians treating the many disabling symptoms of IBS. However, worry about potential side effects, the need for long-term medication and high drug costs have been a deterrent for many IBS patients. Melatonin is one of the drug that was identified as potentially useful in IBS especially for pain symptom as well as bowel motility in constipation predominant IBS.

Melatonin (N-acetyl-5-methoxytryptamine), a hormone produced by the pineal gland, has been studied as a potential treatment of circadian rhythm sleep disorders, cancer, immune disorders, cardiovascular diseases and insomnia[8,9]. The melatonin signal chemically regulates the sleep-wake cycle by causing drowsiness and lowering body temperature[10]. The gastrointestinal tract is another large source of endogenous melatonin.

## MELATONIN IN GASTROINTESTINAL TRACT

Melatonin is produced by enterochromaffin cells of the digestive mucosa. There is higher concentration of melatonin in the gastrointestinal tract than the blood or the pineal gland. The finding that the concentration of melatonin in the gastrointestinal tissues surpasses that in the blood by 10-100 times[11] suggests that melatonin may play an important role in the digestive system. Circadian variation of gastrointestinal (GI) melatonin does not appear to be controlled by photoperiodicity (like the pineal gland), but by eating and food composition. A sharp increase in the content of melatonin in GI tract tissue and circulation in response to food intake was reported in volunteers[12,13]. Melatonin played several pivotal local intestinal functions: (1) Regulation of GI motility: Melatonin exerts both excitatory and inhibitory effects on gut smooth muscles. The precise mechanism through which melatonin regulates gastrointestinal motility is still not very clear. Small doses of melatonin accelerated the intestinal transit in rats, while high doses reversed this effect[14]. In one study focusing on gastric emptying, melatonin partially inhibited gastric motility by activating sympathetic neurons. In the stomach, melatonin also reduces nitrergic myenteric innervation[15]; (2) Anti-Inflammatory Reaction: It increases natural killer cell activity and Th2 cell mediated immune responses[16]. Melatonin was shown to reduce the severity of intestinal inflammatory pathologies such as colitis in animal models[17]. Melatonin had also been shown to scavenge reactive oxygen species and inhibit macrophage by suppressing proinflammatory gents including inducible nitric oxide synthase and cyclooxygenase-2[18-20]; and (3) Moderation of Visceral Sensation: Melatonin may also be involved in mediating gut visceral sensation because patients with functional abdominal pain are reported to have a lower urinary excretion of 6-sulphatoxy melatonin and to exhibit a circadian rhythm of lower amplitude compared with healthy controls[21].

Melatonin might be a candidate for IBS treatment based on the following considerations: (1) melatonin has analgesic effects which may help to alleviate abdominal pain and influence the sensation of abdominal distention in IBS patients; (2) melatonin has regulatory effects on gastrointestinal tract motility and sensation which may improve the bowel habits and alleviate abdominal pain or distention in IBS patients; (3) melatonin could have a sleep promoting effect which may useful to treat the sleep disturbance of IBS patients; and (4) melatonin has mood regulation and anti-stress effects which could help alleviate the abnormal psychological parameters observed in IBS patients. Thus, we believe that melatonin might serve the several aspects of IBS treatment strategy because it targets not only the psychological component, *i.e.*, stress, anxiety, depression and sleep disorder but also the peripheral elements of abnormal bowel sensation and motility. Below we examine the possible mechanisms of melatonin in the treatment of IBS.

## PHARMACOLOGY OF MELATONIN

Melatonin is commonly given orally though it also can be given *via* intravenous, intranasal or transbuccal routes. Melatonin is readily absorbed when it is administered *via* any route. It crosses all morphophysiological barriers, *e.g.*, blood-brain barrier and placenta, with ease.

The absorption and bioavailability of melatonin varies widely. When given by mouth, peak melatonin concentration occurs within an hour and serum half-life is approximately 35-50 min[22]. Because of its fast clearance, regular melatonin formulations can produce physiological levels for only 2-4 h[23]. The typical dose range in studies of melatonin's effects on sleep disturbance has been between 0.3-5 mg, with 2-3 mg commonly being used. Ingested melatonin that did not undergo first-pass metabolism in the liver is eventually metabolized, mainly in the liver. After conjugation with sulfuric or glucuronic acid, it is excreted by the kidneys. A single night-time dose is cleared by the following morning. Legal availability of melatonin varies in different countries from over the counter in United States to prescription only in other countries. It is categorized by the United States Food and Drug Administration as a dietary supplement. Melatonin treatment has an extremely wide margin of safety though

it may cause minor adverse effects, such as headache, rash and nightmares. Studies of human subjects given varying doses of melatonin (1-6.6 g/d) for 30-45 d did not reveal abnormalities at the end of the test period except drowsiness[24,25]. However, in a placebo-controlled trial using 3-6 mg of melatonin for eight weeks on IBS women, drowsiness only happened in a minority of participants and there was no difference between the groups[26,27]. Lu *et al*[26] also showed that baseline saliva melatonin levels were lower in IBS compare to normal control and oral melatonin supplement was able to increase the level of melatonin in the saliva.

## WHAT ARE THE PUTATIVE SITES OF ACTION OF MELATONIN IN IBS?

### *Sleep promoter*

Besides the bowel symptoms, sleep disturbance is commonly observed in patients with IBS, it being reported to occur in 26%-55% of IBS patients[28-30]. Although the cause and effect association is not clear, there is some evidence supporting the "bad bowels cause bad dream" hypothesis[31-36] including the finding that IBS patients have more frequent rapid eye movement (REM) sleep - a sleep phase that is characterized by arousal - than non-REM sleep[37,38]. IBS patients were also found to have higher rapid eye movement latency[34,39]. IBS patients with sleep dysfunction were also found to have abnormal physiological threshold of pelvic muscles. IBS patients had a significantly lower threshold volume for urge and anal sphincter pressure for maximal squeeze as compared with those without sleep dysfunction[40].

It has been suggested that melatonin has a sleep promoting effects by cueing circadian rhythms and thus indirectly promoting sleep[41]. In addition, melatonin was also suggested to have a role in direct promotion of sleep[42]. Currently, there is a general agreement that melatonin is probably not a direct soporific or hypnotic compound[43]. Rather, the most commonly proposed mechanism for melatonin to induce sleepiness relates to its effects on the circadian clock, *i.e.*, it "opens the sleep gate"[44] and also it slightly reduces body temperature which promotes sleep[45]. Clinical trials in healthy volunteers have shown that exogenous melatonin accelerates sleepiness probably *via* thermoregulatory mechanisms[46]. Melatonin has these effects over a wide range of doses, ranging from physiological (250 μg) to pharmacological (1-10 mg) levels[40]. Besides the above effects of melatonin on sleep in healthy subject or animals, many clinical trials and reviews have shown that melatonin may exert sleep promoting effect in a number of circadian rhythm sleep disorders[20,47-49].

### *Brain-gut interaction: mood enhancer*

Patients with IBS often complain of a wide variety of symptoms apart from GI symptoms, which may not necessarily originate from the GIT but from central abnormal psychological conditions such as stress, anxiety and depression. Psychological distress and major life events are frequently present in IBS. The most common comorbid psychiatric disorders seen in IBS patients include anxiety disorders (panic and generalized anxiety disorder), depression, somatoform disorders and phobic disorders[50-52]. Compared with healthy controls, patients with IBS are observed to have higher scores for anxiety, depression, hostile feelings, sadness and interpersonal sensitivity[53-55]. In United States, Whitehead *et al*[56] reported a prevalence of 30.5% for depression and 15.5% for anxiety state in IBS patients. IBS symptoms are often exacerbated by psychological stress. In Hong Kong, Generalized Anxiety Disorder was five times more common among IBS patients than non-IBS control[57].

Melatonin is documented to have a possible role in regulation of mood disorders, such as anxiety and depression, both of which are often caused by certain acute or chronic stress events[52,58]. Many studies reported decreases in nocturnal melatonin concentrations in depressed patients, compared with controls[19,59]. Antidepressant therapy has been reported to restore the circadian melatonin rhythm in depressive patients[60]. It was observed that most melatonin treated women reported a general improvement in mood and a significant mitigation of depression[61]. Reduction of nocturnal melatonin peak has been observed in depressed patients in most studies and an increase in nocturnal melatonin levels has been found in patients during treatment with desipramine[52,62].

Clinical studies in IBS patients with melatonin had mixed result when it comes to depression and anxiety. Two studies in Singapore using 3 mg of melatonin showed that there was no difference in depression and anxiety score in subjects taking melatonin compared to placebo[23,63]. Another study in India showed improvement of psychological well-being and mood in the treatment group taking 3 mg of melatonin for 2 wk, however, the details of psychological parameters were not provided[24].

### *Antinociceptive action of melatonin*

The clinical finding that patients suffering less from pain during the night when melatonin level is higher led to the suggestion that melatonin has a possible analgesic effect. This suspicion was supported by the finding that pinealectomy abolished such dark phase analgesia[64] and that it could be restored using melatonin replacement[65]. However, the mechanism of the analgesic effects of melatonin is still not clear at present. It may include complex interactions among melatonin, opioidergic system and melatonin receptors. Met-enkephalin and beta-endorphin are two endogenous opioids involved in the regulation of pain sensitivity in hypothalamus. The levels and circadian rhythmicity of these two opioids changed in rats that received pinealectomy[66,67]. This may imply that the change in the brain concentration of these endogenous opioids could be a mechanism for the mediation of the melatonin induced modulation of pain sensitivity. However, a recent study found that melatonin exerts its analgesic actions not by binding to opioid receptor subtypes but by

**Table 1 Placebo-controlled studies: treatment effect of melatonin in irritable bowel syndrome patients**

| Ref. | Subjects (total, age, IBS Criteria) | IBS subtypes | Treatment *vs* control | Pain | Bloating/ distention | Motility | Sleep | Psycho-logical | Overall IBS score | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|
| Song *et al*[63], 2005, Singapore | 40, 20-64 years old, ROME Ⅱ IBS (with sleep disturbance) | 14 IBS-C, 18 IBS-D, 8 IBS-A | 20 (3 mg, bedtime, 2 wk) *vs* 20 Placebo | Yes[4] | No | No | No | No | N/A | Decreased abdominal pain and increased pain threshold |
| Lu *et al*[26], 2005, Singapore | 17, 41+/-14 years old woman, ROME Ⅱ IBS | N/A | 12 (3 mg bedtime for 8 wk) *vs* 12 Placebo | Yes[5] | Yes[5] | No[1] | No | No | N/A | Effective in improving bowel symptoms in female IBS patients |
| Saha *et al*[27], 2007, India | 18, 18-65 years old, ROME Ⅱ IBS | N/A | 9 (3 mg bedtime for 8 wk) *vs* 9 Placebo | Yes | Yes | Yes | N/A | Yes | Yes[2] | Improved overall IBS score, extracolonic score as well as QOL |
| Chojnacki *et al*[72], 2013, Poland | 80, 48-65 years old woman, ROME Ⅲ IBS | 40 IBS-C, 40 IBS-D | 40 (3 mg morning, 5 mg bedtime for 6 mo) *vs* 40 placebo | N/A | N/A | Yes[3] | N/A | N/A | Yes[3] | Improved visceral pain and abdominal bloating for IBS-C patients |

[1]CTT significantly prolonged in control subjects. Only a trend of prolonging CTT in IBS patients; [2]Improved overall IBS score (45% *vs* 16.66%, $P < 0.05$); [3]Significant result only for IBS-C, the intensity of visceral pain and abdominal bloating had decreased in 70% of patients ($P < 0.01$) and constipation in 50% of patients ($P < 0.05$); [4]melatonin taken for two weeks significantly decreased mean abdominal pain score (2.35 *vs* 0.70; $P < 0.001$) and increased mean rectal pain threshold (8.9 *vs* 21.2 mmHg; $P < 0.001$); [5]The improvement in mean ± SD. IBS symptom score was significantly greater after treatment with melatonin (3.9 ± 2.6) than with placebo therapy (1.3 ± 4.0, $P = 0.037$ The beneficial effects of melatonin were most marked in symptoms such as abdominal plain, abdominal distension and abnormal sensation of defecation. CTT: Colonic transit time; N/A: Not available; IBS: Irritable bowel syndrome; QOL: Quality of life.

**Table 2 Placebo-controlled studies: side effects of melatonin in irritable bowel syndrome patients**

| Ref. | Subjects (total, age, IBS criteria) | Dosage, frequency, duration | Sleepiness | GI side effect | Others |
|---|---|---|---|---|---|
| Lu *et al*[26], 2005, Singapore | 17, 41+/-14 years old woman, ROME Ⅱ IBS | 3 mg bedtime for 8 wk | 1 × Daytime sleepiness (both treatment and placebo group) | Nil | Nil |
| Saha *et al*[27], 2007, India | 18, 18-65 years old, ROME Ⅱ IBS | 3 mg bedtime for 8 wk | 1 × Drowsiness (both groups), | Nil | 1 decreased libido |
| Chojnacki *et al*[72], 2013, Poland | 80, 48-65 years old woman, ROME Ⅲ IBS | 3 mg morning, 5 mg bedtime for 6 mo | Nil | Nil | 2 fatigue, 1 vertigo |

IBS: Irritable bowel syndrome; GI: Gastrointestinal.

binding to its own receptors and increasing the release of beta-endorphin[68]. Another study also showed that of the three other subtypes of melatonin receptors identified, *i.e.*, Mel1, Mel2 and Mel3, only Mel2 receptor is involved in the analgesic activity of melatonin[69]. Importantly, this anti-nociceptive effect may be unrelated to and independent of the sleep-inducing effects of melatonin, as was demonstrated in the study by Song *et al*[63], where melatonin was found to increase rectal pain thresholds but had no significant effect on sleep. Human studies have shown that melatonin is a hormone with potential therapeutic use for treatment of diseases with pain. Melatonin was documented to be effective in treating many types of headache, such as chronic cluster headache and migraines[70,71].

All these evidence support the belief that melatonin is involved in the modulation of pain and has analgesic effects. However, its potential as a therapeutic agent for treatment of diseases with pain still needs to be further investigated.

### *Outcomes in placebo-controlled studies*

Placebo-controlled studies in melatonin suffered from heterogeneity in methodology (Table 1). Most studies utilized 3 mg at bedtime as the standard dose of trial. The duration of investigation also differ from 2 wk to 6 mo. Chojnacki *et al*[72] used a twice a day dosing in their study with 3 mg in the morning and 5 mg at night for 6 mo. However, there was no increased sleepiness or gastrointestinal side effects reported (Table 2). There was a variety of outcome measures from overall IBS score to quality of life. Lu *et al*[73] examined the effect of melatonin on colonic transit time (CTT) and found that melatonin increased CTT in both control and IBS patients, but only the result in control subjects was significant statistically. In other hand, Chojnacki *et al*[72] showed that with 6 mo treatment with melatonin, 50% of IBS-C patients showed improvement of constipation. However, all studies had consistently showed improvement in abdominal pain for IBS patients. Song *et al*[63] also reported increase of rectal pain threshold after 2 wk of melatonin treatment. Finally, Saha *et al*[27] showed that the overall improvement in quality of life score was 43.63% in melatonin group and 14.64% in placebo group.

## CONCLUSION AND THE FUTURE FOR MELATONIN IN IBS

It is still unclear how melatonin may be useful and its mode of action in IBS patients. Current available evi-

dence showed that it is likely to be useful in battling the pain and increasing pain threshold in IBS patients. Different dosing as well as treatment period of melatonin should be studied. Melatonin is a relatively safe drug that possesses potential in treating IBS. Its attractiveness also stem from its relative low cost to the patients. Future studies should focus on melatonin effect on gut mobility especially in IBS-C patients as well as its true central nervous system effect in view of high placebo rate often observed in IBS patients.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

# Irritable bowel syndrome: The evolution of multi-dimensional looking and multidisciplinary treatments

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## Abstract

Irritable bowel syndrome (IBS) is common in the society. Among the putative pathogeneses, gut dysmotility results in pain and disturbed defecation. The latter is probably caused by the effect of abnormal gut water secretion. The interaction between abnormal gas accumulation, abdominal pain and bloating remains controversial. Visceral hypersensitivity and its modification along with the central transmission are the characteristics of IBS patients. The identification of biologic markers based on genetic polymorphisms is undetermined. Imbalanced gut microbiota may alter epithelial permeability to activate nociceptive sensory pathways which in turn lead to IBS. Certain food constituents may exacerbate bowel symptoms. The impact of adult and childhood abuses on IBS is underestimated. Using the concept of biopsychosocial dysfunction can integrate multidimensional pathogeneses. Antispasmodics plus stool consistency modifiers to treat the major symptoms and defecation are the first-line drug treatment. New drugs targeting receptors governing bowel motility, sensation and secretion can be considered, but clinicians must be aware of their potential serious side effects. Psychiatric drugs and modalities may be the final options for treating intractable subjects. Probiotics of multi-species preparations are safe and worth to be considered for the treatment. Antibiotics are promising but their long-term safety and effectiveness are unknown. Diet therapy including exclusion of certain food constituents is an economic measure. Using relatively safe complementary and alternative medicines (CAMs) may be optional to those patients who failed classical treatment. In conclusion, IBS is a heterogeneous disorder with multidimensional pathogeneses. Personalized medicines with multidisciplinary approaches using different classes of drugs, psychiatric measures, probiotics and antibiotics, dietary therapy, and finally CAMs, can be considered.



**Key words:** Antispasmodics; Biopsychosocial dysfunction; Comorbidity; Genetics; Irritable bowel syndrome; Microbiota; Probiotics; Visceral hyperalgesia

**Core tip:** Irritable bowel syndrome (IBS) is common in the society. Patients with this disorder have a poor quality of life with severe impact on their social and economic burdens. Its pathogenesis remains evolutional, involving biological, psychiatric and social factors. Therefore, the biopsychosocial dysfunctional model has attempted to integrate all the above mentioned mechanisms in order to understand how IBS can develop under such complex interaction. Since the etiology of IBS is heterogeneous, the currently recommended treatments are multidisciplinary and also individualized, *e.g.*, using different classes of drugs, psychiatric measures, probiotics and antibiotics, dietary therapy, and finally complementary and alternative medicines.

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## INTRODUCTION

Irritable bowel syndrome (IBS) is an essential member of the functional gastrointestinal disorder (FGID) family. According to the globally accepted Rome Ⅲ definition, it is characterized by chronic and recurrent abdominal pain/discomfort associated with disturbed defecation[1,2]. As a functional disorder, IBS definition remains evolutional over recent decades. For example, the Manning criteria released in 1978 are just to identify IBS. The later released Rome Ⅰ-Ⅲ criteria are broadly to diagnose all FGIDs including IBS. Now the Rome Ⅳ criteria are undergoing preparation but not formally announced[3]. Regarding various criteria, a review indicated that the Manning criteria are the most valid and accurate, whereas the Rome Ⅲ criteria are not valid and are poorly adopted, especially for clinical trials[4]. It is also controversial whether abdomen pain is virtually required to diagnose IBS. For example, constipation-predominant IBS (IBS-C) and functional constipation are two exactly distinct FGIDs because the latter lacks obvious abdominal pain, but a study indicated that their discrepancy was not easily to detect since marked overlapping was observed between the two conditions[1,5]. Accordingly, an expert meeting recommended that current criteria to diagnose IBS need further revision, particularly the significance of abdominal bloating should be included and the pain component is best to de-emphasize[6]. Overall, IBS is common in the society with worldwide prevalence ranging from 5% to 15%[3,7-10]. The reported IBS prevalence is determined by a number of factors including subject gender, used criteria, questionnaires, study methods, locations, geographical characters, cultural and social backgrounds, and ethnicity[3,8,9,11]. Clinically, IBS is not only confined to the colon but may also extend to other organs and systems since IBS individuals usually have multiple comorbidities such as dyspepsia, gastro-esophageal reflux disease, interstitial cystitis, fibromyalgia, chronic fatigue, insomnia, headache/migraine and psychiatric disturbances[12-18]. Owing to the commonly associated somatic comorbidities and high level of psychiatric disturbances, IBS subjects often have absenteeism, reduced quality of life (QoL) and multiple healthcare seeking behaviors, which lead to great social and economic burdens[13,16,19-21]. Because IBS is a functional disorder with multi-dimensional looking, current IBS management is towards multidisciplinary approaches[1,7,22-24]. The purpose of this review attempts to introduce what are the updated pathogeneses and managements of IBS based on the multi-dimensional looking and multidisciplinary approaches.

## PATHOGENESIS OF IBS

### *Biopsychosocial model*

Current mechanisms to address IBS pathogenesis consist of the defects involving biological, chemical, physical, environmental, economic, cultural, moral, and spiritual events, particularly these defects may interact with each other and lead to IBS. Overall, these mechanisms can be simply categorized into three major issues in terms of biological defects, psychological disturbances and social impacts[1,25]. In order to illustrate and understand why a disease or disorder will develop under the complex interaction involving many mechanisms, the framework of a biopsychosocial model has been introduced to unify biological, psychological and social issues together to indicate their final interaction[25]. Figure 1 briefly depicts that the original existence of any defects among three categories during the early life and adolescent period may initiate biopsychosocial interaction and the following IBS symptoms.

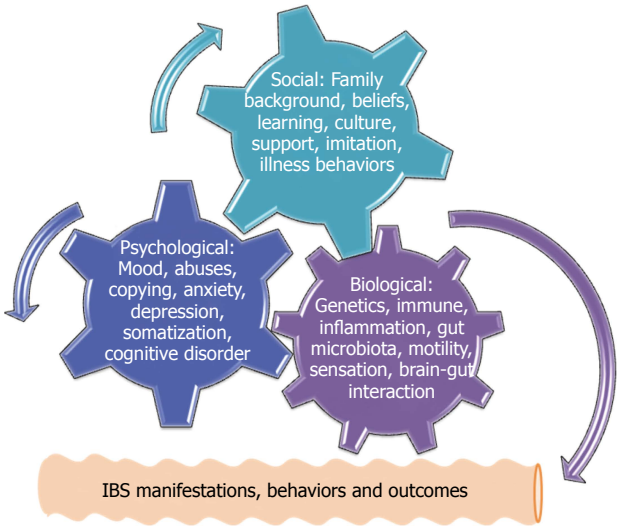


**Figure 1 Three-axis cogwheel system to illustrate how biopsychosocial dysfuntion can integrate many putative pathogeneses leading to irritable bowel syndrome.** The irritable bowel syndrome (IBS) clinical manifestations, disease behaviors and future outcomes are also under the impact of this dysfunction.

Alternatively, genetics- or environment-determined biological defects at any level of neural control and modulation of gut motility, digestion, sensation, endocrine, secretion and immune functions may result in IBS symptoms, while the psychological disturbances which are closely related to a number of social impacts such as early life abuses, stresses, social learning, and copying patterns are able to trigger neuroimmune reactions *via* the brain-gut axis and lead to exacerbated IBS symptoms[25]. Most importantly, the biopsychosocial model is characterized by bi-directional causality and feedback. Accordingly, any adolescent modification coming from the biological, psychological and social impacts manifests different levels of symptoms, behaviors and outcomes of IBS in adulthood, while their symptomatic manifestations are in turn to modify the existent psychological and social events[1,25-27]. This is why many associated comorbidities are reported among IBS subjects. Interestingly, the biopsychosocial model is not only confined to the IBS but

also adopted in many pain related disorders such as migraine, tension headache, chronic fatigue syndrome, and fibromyalgia[28,29]. Thereafter, a concept of central sensitivity syndromes is proposed to unite these comorbidities that apparently share the same biopsychosocial dysfunction[30,31]. Although the unified biopsychosocial model can help easily to understand how IBS will develop under this interaction, the individual pathogeneses are worthy of being introduced before their final integration.

### Motility disorders

Based on the predominant defecation pattern, various IBS subtypes are traditionally defined[1]. Accordingly, it is reasonable to speculate that bowel dysmotility may result in IBS, particularly the disordered defecation. For instance, abnormal small intestinal motility was indicated to lead to IBS in some subjects[32]. In addition, rapid small intestinal transit among the diarrhea-predominant IBS (IBS-D) subjects and delayed transit in IBS-C subjects were reported[33,34]. Using ingested radiopaque markers to count scattering index representing small intestinal transit, another study pointed out the same transit among three categories in terms of IBS-C, IBS-D and control subjects[35]. Accordingly, observed small intestinal dymotility is likely to exist in certain IBS subjects, but the intra- and inter-individual variations in motility measurements limit their interpretation of small intestinal dysmotility in clinical usefulness[36].

Defecation is a complex event involving the coordination of colon transit, high amplitude propagated contractions (HAPC) and pelvic floor synergia while the integrated central (CNS), autonomic and enteric nervous systems (ENS) are virtually required to mediate their correct process[37-39]. Abnormal colon motilities have been observed among IBS subjects. For example, the total colonic transit time in IBS-D patients measured by ingested radiopaque markers was prolonged after pinaverlum bromide treatment. The effectiveness of this agent to treat IBS-D appears *via* correction of abnormal colon transit[40]. Similarly, a radiopaque study confirmed again that Japanese IBS-D subjects had accelerated colon transit compared to controls, whereas those in IBS-C subjects and controls were the same[35]. Left colonic segmentation pressure waves and HAPC were altered among some non-IBS-C patients[41]. Besides, certain IBS-C patients had delayed total colon and right segmental transit[42]. Like small intestinal transit, it is concluded that abnormal colon transit probably exists in some, but not all IBS subjects, because IBS is heterogeneous in its pathophysiology.

Regarding the autonomic nervous activity, IBS-D subjects manifested an enhanced adrenergic sympathetic dominance compared with controls and IBS-C subjects, while this dominance was likely the effect of vagal withdrawal rather than true enhancement[43]. As one of mechanisms leading to functional constipation, pelvic floor dyssynergia was also observed in some non-IBS-D patients[34,44]. Since pelvic floor dyssynergia obviously overlaps with the spectrum of functional anorectal disorder defined by the Rome Ⅲ criteria[25], it is debatable what is the demarcation between IBS and functional anorectal disorder. Overall, colonic dysmotility probably exists in certain, but not all IBS patients. Using various colon motility measurements to diagnose IBS may be unreliable.

### Gut water secretion

Gut water component has been a main factor to determine hard or loose stool. IBS subtypes are traditionally classified by the predominant stool pattern. Alternatively, it means that the gut water secretion in IBS subjects may be different. Unlike other mechanisms that are extensively evaluated, only a few studies have addressed this issue. For example, a rat IBS model study pointed out that the fecal water content was lower in IBS-C rats, whereas an excessive secretion existed in the IBS-D group[45]. The densities of some peptides mediating gut motility, secretion and sensation, *e.g.*, serotonin, peptide YY, pancreatic polypeptide, enteroglucagon, somatostatin, *etc.* were obviously reduced in human IBS colon. It looks to mean that the abnormal gut water secretion is one of components leading to IBS[46]. In addition, using lubiprostone with the ability to increase gut water secretion in softening stool for IBS-C subjects appears to support the role of gut water secretion in IBS[47]. Overall, the abnormal gut water secretion should not be forgotten as a candidate of IBS pathogeneses.

### Bowel gas

Both abdominal bloating and fullness are common among IBS subjects. Therefore, abnormal bowel gas accumulation may account for these annoying symptoms[6]. Unfortunately, bowel gas studies report conflicting results. An earlier study did not find abnormal bowel gas accumulation among the very limited IBS like subjects[48]. In contrast, IBS patients had impaired transit and tolerance to the loading of intestinal gas[49]. A Japanese study pointed out the excessive bowel gas volume among IBS subjects. However, neither symptoms nor subtypes correlated well with abnormal bowel gas accumulation[50]. This means that other factors apart from bowel gas may be responsible for the bloating symptom. Alternatively, bloating symptom is additionally associated with visceral hypersensitivity and delayed transit, and the impaired gas handling may be observed in some, but not all IBS subjects[51].

### Visceral hypersensitivity

Abdominal pain has been a key component of IBS. It is expected that visceral hypersensitivity may account for IBS. Studies using rectal balloon distension repeatedly confirmed that IBS subjects have diminished threshold and exaggerated painful severity to balloon distension[41]. Accordingly, visceral hypersensitivity appears a candidate of biological hallmark to diagnose IBS[52]. In fact, hypersensitivity among IBS subjects is not only confined to the colon but also extends upward to CNS[53-55]. For example,

abnormal activation of certain brain regions following painful rectal stimulation confirmed the altered processing of afferent signals along the brain-gut axis[54]. Visceral hypersensitivity is additionally modified by the gender, peptide, immune and emotional factors[14,25,56,57]. The central projection and modulation of visceral pain are complex, and many transmitted tracts have not been clearly revealed. It is believed that prefrontal lobe may modulate the neural activities coming from limbic and paralimbic regions, anterior cingulate cortex, and hypothalamus, which in turn down modifies the activities of descending inhibitory and facilitatory pathways through the periaqueductal gray and pontomedullary nuclei. The neuronal activities among these cortico-limbic pontine networks can coordinate the final perception of cognitional and emotional impacts on the visceral pain and discomfort in IBS subjects[56,58].

Based on the neuroimage technique, IBS subjects were observed to have long-term micro-structural brain changes, particularly the regions integrating sensory information and cortico-thalamic modulation[59]. These observed brain structural changes among IBS patients appear to challenge the concept of IBS as a functional disorder without existing structural abnormality. The altered functional connectivity between brainstem pain-modulating circuits and cortical-limbic centers suggests a bi-directional interaction between pain and mood. Interestingly, this dysfunctional pain network not only exists in IBS but also is observed among other comorbidities, *e.g.*, migraine, fibromyalgia, anxiety disorders, *etc.*[60]. Allodynia is a pain condition originated from a stimulus, which does not normally provoke pain. Alternatively, it is a central hypersensitivity phenomenon with diminished threshold to triggers[61]. Apart from visceral hypersensitivity, IBS subjects also had cutaneous allodynia following a number of repetitive nociceptive thermal stimuli[62,63]. Overall, the broadly existing somatic, visceral and central hypersensitivities support why IBS patients always have multiple somatic and psychiatric comorbidities.

### Genetics

Twins are an ideal model to resolve whether genetics or environmental factor is essential to determine IBS in a family. Unfortunately, the results of twin studies are conflicting. Concordance for IBS was significant among monozygotic (17.2%) twins compared to dizygotic (8.4%) twins[64,65]. In contrast, similar prevalences were reported between monozygotic (17%) and dizygotic (16%) twins[66]. A meta-analysis based on twin studies further indicated that heritability is more significant among migraineurs (50%) compared to IBS subjects (25%)[67]. It means that both environmental factors and learning behaviors, rather than the heredity only, are the necessary determinants leading to IBS. This viewpoint confirms again that IBS is most likely the final result of biopsychosocial dysfunction involving the interaction of genetically determined biological and psychological factors and exposed environmental factors coming from biological, psychological and social events. Of mitochondrial dysfunctions and associated DNA sequence variants of maternal inheritance, 60% were related to bowel dysfunction, whereas 16% were probable non-maternal inheritance. This suggests that defective mitochondrial energy metabolism among matrilineal relatives probably leads to FGIDs including IBS[68]. Overall, genetics may be a factor leading to IBS, but environmental and learning factors are also involved.

There are numerous peptides/substances and their corresponding receptors that are involved in IBS pathogenesis. Their roles are mainly to mediate gut motility, sensation, permeability, secretion and immune response. The most frequently addressed peptides include 5-hydroxytyptamine (5-HT), cholecystokinin, glucagon-like peptide, somatostatin, neuropeptide Y, endocannabinoid, vasoactive intestinal polypeptide, corticotropin releasing hormone (CRH), *etc.*[1,7,23,41,46,69-72]. For example, the fact that 5-HT related agonists and antagonists have been developed effectively to treat either IBS-C or IBS-D patients strongly suggests that certain peptide dysfunction is one of important mechanisms leading to IBS[1,7,23]. Second, human IBS colon was observed to have low densities of gut peptides including serotonin, peptide YY, pancreatic polypeptide, enteroglucagon, somatostatin, *etc.*[46]. Third, CRH has been a main mediator of stress response in the brain-gut axis, while IBS is believed a dysfunctional brain-gut link which can be exaggerated *via* CRH related stress[71].

Peptide abnormalities among IBS subjects are sometimes genetically determined. Accordingly, variation of genotypes or polymorphisms among those genes governing peptide synthesis and metabolism, mucosal ion channel functions, reuptake of neurotransmitters and their optimal functioning in receptors, and inflammation susceptibility may account for the IBS phenotypes and symptomatic severity[73]. Some genetic polymorphisms have been identified in relation to IBS even with impacts on the therapeutic response, *e.g.*, *CRH-R1* gene polymorphism of TT genotype of rs7209436 and rs242924 among Japanese IBS patients, SS genotype of serotonin reuptake transporter polymorphism among Indian C-IBS subjects, mitochondrial adenosine triphosphate 6 and 8 polymorphisms among Chinese IBS-D patients, and serotonin transporter promoter genetic polymorphisms influencing response to alosetron therapy among American IBS-D patients[74-77]. Current IBS candidate genes consist of serotonin transporter (*SLC6A4*), norepinephrine transporter (*NET*), alpha-2A-adrenergic receptors (*ADRA2A*), interleukin-10 (*IL-10*), G protein β3 subunit (*GNβ3*), sodium channel (*SCN5A*), *etc.*[78]. Regarding genes controlling inflammation, a meta-analysis indicated that high producer IL-10 (-1082 G/G) polymorphism diminishes the IBS risk in the European IBS population, whereas tumor necrotic factor (TNF) (-308 G/G) polymorphism increases IBS susceptibility and TNF (-308 G/A) polymorphism decreases IBS susceptibility in the Asian IBS population[79]. Overall, IBS genetic

polymorphism studies are criticized with drawbacks of very limited case number, inconsistent results, lack of reproducibility, heterogeneous nature of IBS, *etc.*, while no single gene is globally confirmed to be responsible to IBS[80]. Nevertheless, genetic polymorphisms or pharmacogenetics open a door using an optimal substance to treat appropriate subjects *via* proper genetic mapping in the future.

### Gut microbiota and immunity

Human fetus is initially sterile before birth and begins to be infected by many microorganisms since birth through the contact with external environment, while the human immune system is gradually maturing to adapt and tolerate the challenge of exposed microorganisms. Among organs with microorganism residence, the colon owns the most number of resided microorganisms[81]. In fact, the colon microbiota provides numerous physiologic events, namely, supplying energy, nutrient accessibility including short-chain fatty acids, enhancing immune and normal homeostasis, influencing organ development such as morphogenesis of the bone and visceral organs, and even the host metabolism[81,82]. Regarding their clinical impact, inflammatory bowel disease has been the consequence of uncontrolled and imbalanced gut microbiota with altered defense system, permeability and immune response[83]. Similarly, dysfunctional gut microbiota may activate mucosal innate immune responses, which in turn increase epithelial permeability, activate nociceptive sensory pathways, dysregulate the ENS, and finally lead to various FGIDs including IBS. For example, a 16S rRNA-based microbiota profiling study demonstrated both quantitative and qualitative changes of mucosal and fecal gut microbiota among IBS subjects[84]. Second, Japanese IBS subjects had much higher counts of *Veillonella* and *Lactobacillus* than controls, while the products of microbiota such as acetic acid, propionic acid and total organic acids were also significantly higher among these subjects[85]. Third, the methanogenic flora in North Indian IBS patients measured *via* lactose hydrogen breath test was lower compared to controls and this observation was suggested to be the nature of flatulence among them[86].

Apart from the suggested alteration in brain gut axis functions, colonic immunological changes such as chronic and low-grade immune activation are reported among IBS patients. The mediators released by these immune responses may have an impact on the functions of gut mucosal permeability and nerves, leading to the further closed interaction between the immune system and the brain gut axis and finally the observed IBS symptoms[87,88]. For example, post-infectious IBS is to address a phenomenon that previous enteritis may be followed with IBS symptoms, particularly the IBS-D seen months later[89]. Briefly, these patients have excessive numbers and increased activation of mucosal immune cells including mast cells and lymphocytes. In addition, releasing factors such as proteases, histamine, and prostanoids attenuate permeability and activate abnormal neural response, leading to abdominal pain and changed bowel habits, which correlate well with IBS symptoms[88,89]. In addition, psychological stress and activation of Toll-like receptors are also involved in the neuroimmune response among these subjects[56,57]. Besides, antibiotic therapy reduced stress induced visceral hypersensitivity, enhanced bacterial wall adherence and increased luminal s-IgA levels in dysbiotic mice[90]. Considered together, emotional stress, gut microbiota and host immune system interact with each other to respond with altered bowel motor, sensory and secretory functions observed among IBS subjects.

### Food

The experience of certain food ingestion and its following abdominal symptoms are common among the population. For example, acute chili ingestion aggravated abdominal pain and burning symptoms of FGID subjects[91]. Regarding the self-reported food elicited bowel symptoms of IBS subjects, most of them believed that certain diets such as beans, apple, flour, and plum could trigger bowel symptoms, particularly those foods rich in carbohydrates, fat, and biogenic amines such as milk, wine and pork, while women reported more intolerable food items than men[92]. On the other hand, an objective study indicated that IBS patients did not consume different food calories and constituents, but they usually tried to avoid diets rich in fermentable oligo-, di-, monosaccharides, and polyols (FODMAP), and their diets often contained low contents of calcium, magnesium, phosphorus, vitamin B2 and vitamin A[93]. Regarding the relationship between ingested food and gut microbiota composition, a recent study observed that IBS subjects consuming a restriction diet with a lower content of fermentable short-chain carbohydrates for 4 wk had adequate relief of bowel symptoms, while the concentration and proportion of luminal bifidobacteria were diminished together[94]. In summary, food owing to its certain components seems to be a factor leading to IBS, but the food intolerance of IBS subjects does not mean food allergy.

### Abuse and separation

Childhood abuses including sexual issue are the significant worldwide health burden. For example, abuse has been a main risk factor leading to health problems including shaken baby syndrome and behavioral regression during the developmental period, while its long-term risks consist of mental health disorders, substance use disorders and chronic physical complains in the later adult life[95]. Unfortunately, both physical and sexual abuses are common and underestimated among IBS patients[96]. In addition, these victims often manifest severe pain perception, psychological distress, and poorer health outcome[97]. Their perceptive pattern was already centrally confirmed *via* advanced neuroimage to show an enhanced nociception[98].

Early life trauma is able to increase future visceral pain perception. Accordingly, maternally separated neonatal rodents are used to create a model to study the

**Table 1 Potential drugs and measures to treat irritable bowel syndrome**

| Category | Functions | Examples |
|---|---|---|
| Antispasmodics | Antagonists of muscarinic receptors and calcium channels of smooth muscle | Cimetropium bromide, dicyclomine, hyoscine butylbromide, mebeverine, otilonium bromide, peppermint oil, pinaverium bromide, trimebutine maleate |
| Antidiarrheals | Agonists of μ-opioid receptors | Loperamide |
| Laxatives | Osmotic, stimulant | Bisacodyl, lactulose, magnesium citrate, magnesium sulfate, polyethylene glycol |
| Bulking agents | Water binding to increase stool bulk | Methylcellulose, psyllium, wheat bran |
| Receptor targeted new drugs | Agonists and antagonists of 5-HT | Alosetron, cilansetron, naronapride, prucalopride, ramosetron, tegaserod |
| | Chloride channel activators | Lubiprostone |
| | Agonists of GC-C | Linaclotide |
| | Antagonists of $NK_1$ receptors | Ezlopitant, TAK 637 |
| | Agonists of κ-opioid receptors | Asimadoline |
| | Agonists of α2 adrenergic receptors | AGN-203818, clonidine, solabegron |
| | Antagonists of $CCK_1$ receptors | Loxiglumide |
| | Agonists of somatostatin receptors | Octreotide |
| Psychiatrics | Tricyclic antidepressants | Amitriptyline, desipramine, doxepin, imipramine, trimipramine |
| | SSRIs | Citalopram, fluoxetine, paroxetine, venlafaxine |
| | Psychotherapy | Biofeedback, cognitive behavioral therapy, dynamic psychotherapy, hypnotherapy, relaxation training |
| Probiotics | To balance gut microbiota | VSL-3, lactobacilli, bifidobacteriae |
| Fecal transplantation | Living microbiota supplement | Through nasogastric tube, enema or colonoscopy |
| Anti-inflammation | Mast cell stabilizers, PAR-2 blockers TRPV receptor type 1 and 4 blockers | Capsazepine, GB88, ketotifen, RN1734 |
| Antibiotics | To inhibit gut microorganisms | Neomycin, rifaximin |
| Miscellaneous | Antinociceptive substance | Melatonin |
| | Bile acid sequestrant | Cholestyramine |
| | To diminish inflammation? To absorb bacteria and enterotoxins? | Diosmectite |
| Food | To enhance immunity? | Kiwifruit |
| Complementary and alternative medicine | Mysterious | Acupuncture, aromatic therapy, ginger, herb drugs, holistic medicine, homeopathy, massage, reflexology |

5-HT: 5-hydroxytryptamine; CCK: Cholecystokinin; GC-C: Guanylate cyclase C; NK: Neurokinin; PAR: Protease-activated receptor; SSRIs: Selective 5-hydroxytryptamine re-uptake inhibitors; TRPV: Transient receptor potential vanilloid.

relationship between early life stress, visceral sensation and depression related disorders including IBS. It was indicated that water avoidance stress increased pain perception and activated somatosensory cortex, periaquaductal gray and hippocampus in the maternally separated rats[99]. In addition, maternally separated rats had significantly increased 5-HT content after colorectal distension[100]. This model also pointed out that the colon of maternally separated rats had elevated circulating levels of interleukin-6 in addition to gut dysfunction[101]. Considered together, neonatal maternal separation appears a stress in rats with exacerbated neurochemical, inflammatory responses, and visceral hyperalgesia in the colon and CNS comparable to IBS subjects. It is of interest whether the neonatal separation story does truly happen in the society leading to IBS. A study to explore the childhood events among IBS adults confirmed that loss and separation during childhood, in the current family and conflicted or dependent maternal relationships were common among some IBS patients[102]. In summary, avoidance of any kind of childhood abuses is necessary to demolish future adult onset of IBS, FGIDs and psychiatric events.

## TREATMENT OF IBS

With regard to IBS treatment, patient-centered approach with a strong and effective communication between patients and clinicians has been emphasized to increase the treatment satisfaction and diminish utilization of health care sources[23,103]. In fact, the development of active drugs to exhibit an efficacy greater than placebo in treating heterogeneous IBS is not easily to achieve, because IBS subjects often experience an excellent efficiency up to 40%-50% to placebo treatment[23,104]. Psychologically, placebo effect is believed the total response of treating expectancy, repetitive administration named conditioning and a non-specific psychological effect supported from givers. Now the placebo effect could be well confirmed in the brain *via* functional neuroimage[54]. Table 1 summarizes the multidisciplinary approaches that are optional to treat IBS.

### *Antispasmodics*

Antispasmodics that can block muscarinic receptors and calcium channels of gut smooth muscle cells have been the oldest drugs to treat IBS for decades because of disturbed bowel motility and its effect on abdominal pain are commonly observed among these patients[1,23,34,105,106]. Unfortunately, their effectiveness and recommended evidence are not fair owing to the trial drawbacks including different IBS definitions, limited case number, inappropriate end-points, evaluation methods, dosing, duration, side effect recording, *etc.*[3,23,106]. Apart from hyoscine butylbromide, the only available antispasmodic in United

States, other marketed antispasmodics include dicyclomine, mebeverine, pinaverium, otilonium bromide, peppermint oil, trimebutine maleate, *etc*.[1,2,23,39,106-108]. Overall, a meta-analysis indicated that antispasmodics are beneficial for IBS patients when abdominal pain is the predominant symptom of subjects attempted to treat[109]. Based on their long-term marketing, antispasmodics remain the first-line drugs to treat IBS but their probable anticholinergic side effects are best to warn before the prescription.

### Antidiarrheals, laxatives and bulking agents

Disordered defecation has been another concern of IBS subjects and normalization of defecation *via* various approaches such as antidiarrheals for IBS-D and laxatives or bulking agents for IBS-C is recommended[1,23,106]. Regarding the IBS-D treatment using loperamide, it is a synthetic opiate derivative with an agonistic effect on μ-opioid receptors but scant opioid CNS effects. Its antidiarrheal effect comes from directly simulating gut water absorption and is further augmented by an antisecretory activity mediated by calmodulin antagonism, a property not shared by other opioids[110]. Loperamide appears the only antidiarrheal recommended to treat IBS-D during the acute or chronic diarrhea[1,7,106,110,111]. Earlier trials already supported its efficacy over placebo in treating stool consistency, urgency, borborygmi and abdominal pain[112,113]. However, a meta-analysis pointed out that it seems to reduce diarrhea but does not relieve abdominal pain among IBS subjects[109].

Laxatives have long been recommended to treat the constipation concern of IBS-C subjects[1,23,106]. Surprisingly, laxatives are not well evaluated whether they do have effectiveness in treating IBS-C, because most clinical experiences are adopted from those of functional constipation treatments. Only a small-scaled study pointed out that polyethylene glycol *vs* placebo improved stool frequency but not ameliorated abdominal pain among IBS-C subjects[114]. Until now, the evidence to recommend laxatives in treating IBS-C remains controversial[23,106].

Bulking agents including natural and artificial fibers are also recommended to treat constipated subjects including IBS patients. Basically, unabsorbed soluble agents such as psyllium and polycarbophil are dissolved and fermented in colon water to form a gel in turn to shorten colon transit time and to stimulate defecation, whereas insoluble agents such as corn fiber and wheat bran have limited change in gut, but they increase fecal mass to help defecation[115]. Reported trials indicated a limited benefit for constipation and no effect to attenuate other IBS symptoms[116]. Furthermore, a meta-analysis did not support its efficacy in treating IBS symptoms including stool frequency, abdominal pain and bloating[109]. According to the types of bulking agents, another meta-analysis pointed out that soluble fibers improve global symptoms, whereas insoluble fibers even exacerbate the clinical outcome[23,115]. As fermentable substances, the commonly reported side effects of bulking agents such as bloating, abdominal distension and flatulence are best to inform before the prescription[23].

### Receptor targeted drugs

Since the end of last century, new drugs targeting receptors known to have pharmacological effects on IBS have been emerging. Of them, 5-HT related drugs including agonists and antagonists are most promising because their efficacies over placebo were critically evaluated based on the high quality controlled trials and finally approved by the authorities[23,106]. For example, IBS-D can be treated using alosetron and cilansetron which have antagonistic activity on 5-HT$_3$ receptors to delay bowel transit, reduce colonic tone and HAPC, blunt gastrocolic reflex and decrease visceral sensation, particularly with obvious therapeutic effect among female patients[7,23,117,118]. Nevertheless, this group should be used with very caution because of the possibility of serious side effects including severe constipation and ischemic colitis. Now they are only restricted to female IBS-D patients when conventional therapies have failed[7,23,106]. Ramosetron is another potent and selective 5-HT$_3$ receptor antagonist that can attenuate abnormal colonic function and abdominal pain in experimental animals. Clinical studies conducted in East Asia confirmed its benefits on abdominal pain/discomfort and bowel habits in both male and female IBS-D patients, but it also had side effect of hard stool. Until now, no ischemic colitis was reported based on a small number of cases exposed to it[23,119].

Regarding IBS-C treatment, tegarserod and prucalopride showed an agonistic activity on 5-HT$_4$ receptor-mediated release of 5-HT from mucosal enterochromaffin cells, which promotes ascending excitatory contraction and descending inhibitory relaxation to enhance bowel motility through a series of chain reactions. Apart from attenuating visceral hypersensitivity, these agonists owing to different affinities with 5-HT$_4$ receptors may account for variable prokinetic potentials and side effects[120-123]. Clinically, 5-HT$_4$ agonists diminish bloating and abdominal pain/discomfort with the improved satisfaction to defecation concerns such as stool consistency and straining[23,106,124]. Unfortunately, tegaserod was withdrawn due to serious cardiovascular adverse events. It is indicated that nonselective 5-HT$_4$ agonists such as cisapride and tegaserod may interact with human ether-à-go-go related cardiac potassium channels to have the chance of causing heart arrhythmia, whereas selective 5-HT$_4$ agonists such as prucalopride and naronapride are believed to have cardiovascular safety[123]. Tegaserod was reintroduced in United States in 2007 under a limited and restricted using for women younger than 55 years and not at risk for cardiovascular events[23,123]. It remains uncertain whether prucalopride can effectively treat IBS-C as tegaserod, although its efficacy was confirmed among functional constipation subjects[106]. Renzapride is a substance to own both activities of 5-HT$_4$ agonist and 5-HT$_3$ antagonist, and its development for IBS-C patients was halted because of the disappointing limited effects in a phase Ⅲ trial[23].

Lubiprostone is a newly approved drug available in United States, United Kingdom and Japan to treat constipated subjects including IBS patients. It is a synthetic bicyclic fatty acid derivative of prostaglandin E1 with the ability to stimulate cystic fibrosis transmembrane conductance regulator (CFTR) dependent chloride channels of enterocytes to increase small intestinal secretion of fluid, mucin and electrolytes and finally to improve bowel functions including defecation[23,46,125]. Lubiprostone is safe and effective to treat constipated subjects, but it has some side effects, with nausea being the most common, followed by diarrhea, abdominal pain, bloating, and even the very rare events of dyspnea and ischemic colitis[23,126,127].

Similarly, linaclotide was marketed in United States and Europe to treat severe constipated patients including IBS patients in 2012[128]. It is a synthetic 14-amino-acid peptide of guanylate cyclase C (GC-C) agonist mainly to increase intestinal fluid secretion and gut transit. Unlike lubiprostone, linaclotide first activates GC-C receptors on the luminal surface of enterocytes to enhance intra- and extracellular levels of cyclic guanosine monophosphate and in turn promote CFTR to secrete chloride and bicarbonate into gut lumen to improve defecation. Interestingly, the activation of GC-C receptors also diminishes visceral pain[23,128,129]. Clinically, linaclotide improves abdominal pain/discomfort, bloating and the defecated symptoms of straining, incomplete defecation and stool consistency of IBS-C patients. Meta-analyses confirmed its superior efficacy over placebo to treat IBS-C and functional constipation[128,130,131]. The most common side effect of linaclotide has been severe diarrhea (20%), thus subjects with a tendency to water and electrolyte imbalance are not indicated. Until now, its long-term safety has not been established yet[128,129,131].

Currently, many new drugs targeting the specific receptors responsible for motility, visceral sensation, gut secretion, neuroimmune and brain-gut axis are being developed to treat IBS. Basically, the key factors in terms of clear mechanisms involving whole pathophysiology, good oral bioavailablity, no CYP dependent metabolism, best once daily, least interaction with food and other drugs, no unwanted metabolites, long-term maintenance ability, good safety records and so forth may determine whether these new drugs can be accepted to treat IBS[132]. Because too many new drugs are under development, only a few examples are briefly introduced here. First, TAK 637 is a selective antagonist of smooth muscle neurokinin 1 receptors that activate intestinal muscle contraction. This agent reduced rabbit abdominal contractions induced by colorectal distension *via* inhibition of neurokinin 1 receptors, mainly located in the spinal cord, and it also reduced colonic transit and defecation in a Mongolian gerbil IBS model. Unfortunately, its development was halted because of serious side events that occurred in two animal species[133]. Second, opioid kappa receptors are located on the cholinergic terminals of ENS with the ability to inhibit cholinergic transmission and gut motility. Asimadoline, an agonist of these receptors, reduces gut wall neurotransmitter releasing to exhibit both analgesic and anti-diarrheal effects[7,132,134]. A recent phase Ⅲ trial on IBS-D patients observed excellent results to treat pain and defecation related concerns such as frequency, urgency and bloating[134]. Third, clonidine initially used to treat hypertension with the commonly reported constipation side effect is a $\alpha 2$ adrenergic receptor agonist. It increased colonic and rectal compliance, and reduced tone, pain, gas sensation and rectal urgency of healthy subjects[135]. A trial also indicated its effect on IBS-D patients with reduced abdominal pain, satisfactory relief of global IBS symptoms and improved disturbed defecation in spite of side effects of drowsiness, dizziness and dry mouth[132,136]. Owing to the obvious CNS effects, clonidine is apparently unable to treat IBS. Other adrenergic agonists such as AGN-203818 and solabegron with the purpose to treat IBS are undergoing evaluation[23].

### Psychiatric approaches

Severe and intractable IBS patients who fail conventional therapy may consider the psychiatric approaches such as anxiolytic agents, antidepressants, cognitive behavioral therapy, dynamic psychotherapy and even hypnotherapy[1,23,106,137]. According to the recommendations, antidepressants are only indicated when abdominal pain is the main concern. Its benefits are likely the central antinociceptive effect plus bowel effect[23,106]. When treating IBS patients using either tricyclic antidepressants (TCAs) or selective 5-hydroxytryptamine re-uptake inhibitors (SSRIs), their symptomatic subtypes should be considered. For example, SSRIs such as paroxetine decrease orocecal and whole gut transit times in IBS-C patients. In contrast, TCAs such as imipramine prolong orocecal and whole gut transit times in IBS-D patients[138]. Meta-analyses indicated that IBS global symptoms are improved using both TCAs and SSRIs no matter its subtypes while SSRIs are more tolerable than TCAs owing to their obvious prokinetic effect, but their long-term safety remains unknown[23,106]. Other psychiatric measures are also recommended to treat intractable IBS. Overall, the drawbacks of these non-drug approaches include expert dependence, being unable to have blinding studies, methodological deviation and scant clinical experiences among most gastroenterologists. Nevertheless, experts recommended its good global symptom improvement and less adverse events[1,23]. It may be employed to severe and intractable subjects when all available and conventional treatments have failed.

### Probiotics and antibiotics

Since an abnormal composition of gut microbiota exists among IBS patients, modification of gut microbiota components through exogenous supplement or inhibition of them using antibiotics appears promising to treat IBS patients[81,139]. Probiotics prepared as empiric base of "immune-boosting and health-enhancing" for century are live microbial supplements in attempt to improve gut microbial balance[81,140]. Pharmacologically, the benefits

of probiotics consisting of anti-pathogenic ability *via* secretion of bacteriocins, acidification of the colon by fermentation, anti-inflammation to protect gut mucosa, alteration of mucosal response to stress, barrier enhancement, immune-modulating effects, and inhibition of visceral hypersensitivity justify their use to treat IBS[141,142]. Unfortunately, the worldwide probiotic preparations are not standardized. The most commonly used strains and species include *Streptococcus thermophilus, Lactobacillus rhamnosus Lc705, Bifidobacteria, Lactobacillus rhamnosus GG, L., Bifidobacterium animalis ssp., Lactis Bb12,* and non-pathogenic yeasts such as *Saccharomyces boulardii*. However, no two preparations are the same and the extrapolation of therapeutic responses from one to another may be problematic[23,142,143]. It was indicated that probiotic cocktail had potent anti-inflammatory properties of suppressing mucosal inflammation and restoring cytokine balance[143]. Overall, probiotics are safe without serious side effects but the benefit magnitude and the most effective species or strains are undetermined. Multi-species preparations are probable the best to treat IBS[23,84,143-145].

Live fecal microbiota transplantation is an incredible approach to treat various bowel diseases including inflammatory bowel disease, *Clostridium difficile* infection and even IBS. The fecal content can be administered *via* nasogastric tube, enema and colonoscopy[146]. Limited data indicated that constipated patients treated with colonoscopically delivered fecal microbiota had immediately improved defecation, bloating and abdominal pain[147]. It is unknown whether it is applicable to IBS-C subjects. Apart from microorganism supplement, new drugs targeting colonic low-grade inflammation are being developed, *e.g.,* mast cell stabilizer, transient receptor potential vanilloid receptor type 1 and 4 blockers, protease-activated receptor 2 blockers, *etc.* It appears too early to predict their chance of success[7,132].

Antibiotics provide another route to treat imbalanced gut microbiota. For example, rifaximin has been proved in several non-diarrhea IBS controlled trials to improve global symptoms, abdominal pain, dysfunctional defecation and bloating[23,148,149]. Regarding IBS-C patients, neomycin treatment improved global symptoms and constipation. The success of this treatment depended upon the presence and post-treatment elimination of methane[150]. Owing to the chronic and recurrent nature of IBS, the effectiveness and safety of long-term or repeated use of antibiotics to treat IBS remain controversial.

### Food therapy

Food restricted approaches such as avoidance of FODMAP items and individual evaluation of the effects of protein-, fat- and carbohydrate-rich/poor diets are recommended to reduce some IBS symptoms[84,93,94]. Likewise, a fermentable short-chain carbohydrates restricted diet significantly improved IBS symptoms of United Kingdom patients[94]. In contrast, another study indicated that dietary manipulation of poorly absorbed short-chain carbohydrates increased total amount of gut gas including hydrogen production to exaggerate the bowel symptoms of Australia IBS patients, thus avoidance of this food constituent is recommended[151]. Food elimination towards IgG antibodies in certain IBS patients effectively reduced bowel symptoms[152,153]. Interestingly, kiwifruit is a natural remedy to own laxative ability, particularly among the elderly population[154]. A study found that 4-wk kiwifruit consumption diminished colon transit time, increased defecation frequency, and finally improved the bowel function of IBS-C subjects[155]. Since kiwifruit may support the immune function to reduce the occurrence and severity of flu-like illness, it is unknown whether its efficacy to treat IBS is relevant to enhanced gut immunity[156]. Overall, the restriction of certain diets may be recommended to all IBS patients, but the routine use of food restriction or supplement without an appropriate drug therapy may not be perfect.

### Miscellaneous agents

The intensity of pain perception is usually lower during the night dark hours when blood melatonin level is higher. Consequently, melatonin is considered an antinociceptive substance with the mechanisms broadly involving opioid, benzodiazepine, α(1)- and α(2)-adrenergic, serotonergic, cholinergic and melatonergic (1) and (2) receptors[157]. A short-term oral melatonin treatment improved abdominal pain, distension and abnormal defecation sensation in female IBS patients, whereas the defecation frequency and stool consistency were not affected[158]. Bile acid malabsorption is common among chronic diarrhea subjects and even IBS-D patients. A meta-analysis indicated that this event might be underestimated since about a third of IBS-D patients had moderate to severe bile acid malabsorption[159]. This may explain why cholestyramine is recommended to treat IBS-D patients[1,160]. Mesalazine was observed to reduce the number of mast cells and the subsequent release of mediators and diminish gut permeability and sensitivity in IBS patients, thus a large-scale mesalazine trial is undergoing in an attempt to know whether it can treat IBS-D patients. The final results are expected toward the end of 2013[161]. Diosmectite is inorganic aluminomagnesium silicate clay with a strong adsorbent ability. It is used to treat acute watery diarrhea based on the suggested mechanisms to diminish inflammation and mucolysis, to modify mucus rheologic and to adsorb bacteria, enterotoxins, viruses and other potentially diarrheogenic substances[162]. With regard to IBS treatment, diosmectite diminished abdominal pain and bloating intensity in IBS-D patients, but its effect on the disturbed defecation was not observed[163].

### Complementary and alternative medicines

Traditionally, complementary and alternative medicines (CAM) is a medical practice not belonging to the current conventional medicine with therapeutic effects determined by the cultural, ethnic, social, religion, education and economic backgrounds. The CAM theories are markedly deviated from the conventional medicine in terms of

heterogeneity, disease mechanisms, diagnostic approaches, therapeutic measures, judging efficacies, *etc.*[164,165]. Now herb drugs based on Chinese, Indian, Ayurvedic, and Tibetan preparations, acupuncture, aloes, aromatic therapy, ginger, homeopathy, probiotics, peppermint oil, reflexology, massage, colon irrigation, holistic medicine, aromatherapy, Qi gong, bioelectromagnetic field therapy, *etc.* are categorized as CAM[166]. Interestingly, certain CAM members have been acknowledged by the conventional medicine to treat IBS, *e.g.*, probiotics and peppermint oil.

Clinically, many IBS patients do seek CAM before they encounter clinicians[167]. Herb drugs are the most often used but their effects are conflicting. In fact, the therapeutic effects of herb drugs are very hard to evaluate and compare each other since they are criticized with the drawbacks of mixture of variable botanical components, neither purified nor quality control, lack of preclinical animal study, unique preparation as family secret, publication bias, no reported adverse events, absent negative reports, *etc.*[23,168]. For instance, a trial conducted on Australian Caucasians with IBS indicated the very promising effect over placebo in relieving bowel symptoms even after discontinuation[168]. In contrast, herb mixture to treat Chinese IBS patients residing in Hong Kong did not reveal any benefits judged by global symptom and individual bowel symptoms[169]. It is unknown whether certain herb drugs claimed effective to treat IBS have the true pharmacological effect or just enhanced placebo response.

Acupuncture is a well-known old Chinese traditional medicine. Basically, it exhibits the physiological impacts on neural, humoral, opioid and serotonegic pathways with the effects of normalized motility, inhibited acid output, antinociceptive effect, reduced rectal hypersensitivity and altered 5-HT functions[170-172]. Acupuncture looks promising to treat FGIDs including IBS. Apart from Chinese studies, the effects of acupuncture to treat IBS among Western people are conflicting. For example, 10 weekly acupuncture sessions compared to placebo procedure for United Kingdom IBS patients reduced their symptomatic severity and its efficacy even persisted over a 1-year period[173]. Another study using 3-wk true acupuncture and cross-over with another 3-wk sham procedure conducted on United States IBS patients did not support its superiority over sham procedure to treat the global symptom and symptomatic intensities[174]. Overall, meta-analyses repeatedly indicated that acupuncture has no effect to the general wellbeing, individual bowel symptoms and QoL of IBS patients[175-177]. Finally, NICE guidance also does not recommend using acupuncture to treat IBS[178].

Homeopathy is popular among the CAM. Unlike conventional medicine, it means that "a substance is capable of inducing a series of symptoms in a healthy living system, and low doses of the same substance can cure these symptoms under certain circumstances"[179]. Homeopathy is claimed effectively to treat IBS. Now a three-arm trial based on 5 sessions of true homeopathic treatment plus usual care *vs* placebo-homeopathy plus usual care *vs* usual care alone is undergoing in United Kingdom and the final result is expected to resolve whether homeopathy is truly effective to treat IBS[180]. Regarding IBS patients who failed all conventional treatments, CAM may be considered as a supplement or alternative with expected efficacy equal to enhanced placebo effect if they do not have any intolerable or serious side effects.

## CONCLUSION

Current Rome Ⅲ criteria-based diagnosis of IBS remains to have limitations, particularly the differentiation from constipation. It probably needs the resolution of coming new criteria. Since IBS is heterogeneous based on the multidimensional pathogeneses, using biopsychosocial dysfunction is effectively to integrate all old and emerging IBS pathogeneses in terms of gut dysmotility, abnormal gut water secretion and gas accumulation, visceral hypersensitivity, impaired mucosal permeability, dysfunctional brain-gut axis, genetic abnormalities, disturbed gut microbiota and immune system, psychological disturbances, impacts from food and various abuses, *etc.* Now multidisciplinary approaches using drugs with different mechanisms of action, imposing psychiatric measures, giving probiotics and antibiotics, possessing diet therapy, and CAM treatment, can be considered individually to treat the major clinical symptoms and other associated concerns.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

# Hepatic inflammation and progressive liver fibrosis in chronic liver disease

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## Abstract

Chronic liver inflammation drives hepatic fibrosis, and current immunosuppressive, anti-inflammatory, and anti-viral therapies can weaken this driver. Hepatic fibrosis is reversed, stabilized, or prevented in 57%-79% of patients by conventional treatment regimens, mainly by their anti-inflammatory actions. Responses, however, are commonly incomplete and inconsistently achieved. The fibrotic mechanisms associated with liver inflammation have been clarified, and anti-fibrotic agents promise to improve outcomes as adjunctive therapies. Hepatitis C virus and immune-mediated responses can activate hepatic stellate cells by increasing oxidative stress within hepatocytes. Angiotensin can be synthesized by activated hepatic stellate cells and promote the production of reactive oxygen species. Anti-oxidants (*N*-acetylcysteine, *S*-adenosyl-*L*-methionine, and vitamin E) and angiotensin inhibitors (losartin) have had anti-fibrotic actions in preliminary human studies, and they may emerge as supplemental therapies. Anti-fibrotic agents presage a new era of supplemental treatment for chronic liver disease.



**Key words:** Inflammation; Fibrosis; Cirrhosis; Treatment; Anti-oxidants; Angiotensin receptors; Investigational drugs

**Core tip:** The prevention of hepatic fibrosis and the reversal of cirrhosis are now achievable objectives in the management of chronic liver disease. Conventional immunosuppressive, anti-inflammatory, and anti-viral therapies can accomplish these outcomes by reducing liver damage, suppressing hepatic inflammation, and eliminating etiological agents, but they do so inconsistently and indirectly. The continuing clarification of pro-fibrotic mechanisms affords opportunities to design site-specific, anti-fibrotic interventions. Anti-oxidants and angiotensin inhibitors have shown promise as adjunctive anti-fibrotic agents in preliminary human studies, and they exemplify a genre of interventions that are likely to influence future management strategies.

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## INTRODUCTION

Hepatic fibrosis is commonly preceded by chronic inflammation[1,2], and persistence of this inflammation has been associated with progressive hepatic fibrosis and the development of cirrhosis[3]. Chronic viral hepatitis and autoimmune hepatitis are chronic inflammatory diseases

of the liver that exemplify this progression, and studies have indicated that prompt and sustained suppression of inflammatory activity by eliminating the etiological agent (virus)[4-10] or dampening the immune response (lymphocytic proliferation and infiltration)[11-15] can halt and even reverse the fibrotic process. The current treatments of chronic liver inflammation were not designed to be anti-fibrotic[9,14], but the success of these treatments in achieving this effect can be measured in prolonged survival and possibly a reduced occurrence of hepatocellular carcinoma[10,16-21].

The molecular pathways that link chronic liver inflammation with progressive hepatic fibrosis continue to be clarified, and this evolving knowledge affords opportunities to directly target the fibrotic process[22-24]. Current conventional treatments that are focused on elimination of an etiological agent or putative immune-mediated mechanism may be supplemented in the future by agents that diminish oxidative stress, dampen hepatic stellate cell activation, reduce myofibroblast proliferation, and increase degradation of the extracellular matrix[22-24]. The challenges are to characterize the disease-specific mechanisms of fibrogenesis, establish the safety and efficacy of the anti-fibrotic interventions in a timely fashion, and incorporate them into conventional treatment regimens.

Highly selective, site-specific, anti-fibrotic therapies are unlikely to replace current treatments for the chronic inflammatory liver diseases, and their eventual emergence into clinical practice is best envisioned as a supplemental therapy[22-24]. Novel anti-fibrotic treatments must have actions that are additive to the anti-inflammatory and immunosuppressive properties that are already possessed by the conventional treatments.

The objectives of this review are to describe the mechanisms by which liver inflammation can stimulate hepatic fibrosis, discuss the putative anti-fibrotic properties of the conventional drug regimens used in the treatment of the chronic inflammatory liver diseases, detail the clinical efficacy of these current regimens, and assess the prospect of ancillary anti-fibrotic therapies.

## HEPATIC INFLAMMATION AS A DRIVER OF HEPATIC FIBROSIS

Liver inflammation is commonly associated with hepatocyte necrosis and apoptosis[1,2,23]. These forms of liver cell injury initiate a sequence of events that is independent of the etiological basis for the inflammation and can result in hepatic fibrosis. Apoptotic bodies derived from the damaged hepatocytes can activate quiescent hepatic stellate cells and Kupffer cells, and these activated cell populations can in turn promote inflammatory and fibrogenic responses[1,2,23] (Figure 1). Transforming growth factor beta 1 (TGFβ1), platelet-derived growth factor, and endothelial growth factor can induce the activated hepatic stellate cells to transform into myofibroblasts[25-33]. The activated hepatic stellate cells can also increase the production of inflammatory chemokines[34], the expression of adhesion molecules[35], and the presentation of antigens to T lymphocytes and natural killer T cells[36]. These enhanced inflammatory and immune-mediated responses can promote hepatocyte necrosis and apoptosis and thereby strengthen and perpetuate the stimuli for fibrogenesis[23,37-40].

Myofibroblasts have a contractile property that is signaled by the expression of α-smooth muscle actin[41]. They are derived from hepatic stellate cells and from portal mesenchymal cells, and their origin may reflect the nature of the liver injury and the microenvironment within the liver. The transition between hepatic stellate cells to myofibroblasts involves signaling pathways, such as Notch and Hedgehog, which modulate the epithelial-to-mesenchymal cell transition[42]. Hepatic stellate cells can be deactivated, and a mesenchymal-to-epithelial cell transition can occur which reverts the myofibroblast to an inactive hepatic stellate cell[42]. This inactive cell remains primed for reactivation, and it may be more responsive to recurrent fibrogenic stimuli than its original quiescent state[41-45]. Deactivation of the hepatic stellate cells terminates fibrogenesis and facilitates regression of the extracellular matrix[43].

The activated Kupffer cells can promote hepatic fibrogenesis by releasing cytokines and chemokines that stimulate the hepatic stellate cells[1,2,23] (Figure 1). The Kupffer cells can also release reactive oxygen species, nitric oxide, and chemotactic proteins that promote hepatocyte injury and nurture the inflammatory response. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase can stimulate the production of reactive oxygen species in hepatic stellate cells, macrophages and hepatocytes[46,47], and the resultant oxidative stress on the hepatocytes can damage deoxyribonucleic acid (DNA), induce apoptosis, promote the expression of pro-inflammatory genes, enhance fibrogenesis, and possibly trigger malignant transformation[47,48]. Inducible nitric oxide synthase (iNOS) can promote hepatocyte toxicity by increasing the production of nitric oxide, and the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) can modulate the production of iNOS and the oxidative stress reaction[49]. The net consequence of these diverse interactive cellular and molecular mechanisms is to perpetuate and extend the tissue injury and enhance the accumulation of the extracellular matrix of collagen[50].

The overproduction and accumulation of collagens Ⅰ and Ⅳ, procollagen Ⅲ, and elastin occur early in liver injury, and metalloproteinases that are directed at the different types of collagen are activated to degrade the depositions and maintain stability of the matrix[23,50] (Figure 1). Tissue inhibitors of the metalloproteinases are also expressed to counter-regulate the degradation process[50,51]. They may also induce expression of the anti-apoptotic protein, Bcl-2, and thereby enhance survival of hepatic stellate cells[23,52]. Maturation of the collagen matrix depends mainly on lysyl oxidases that cross-link the collagen fibrils and increase the resistance to degradation[50,53].

Prevention or reversal of hepatic fibrosis depends

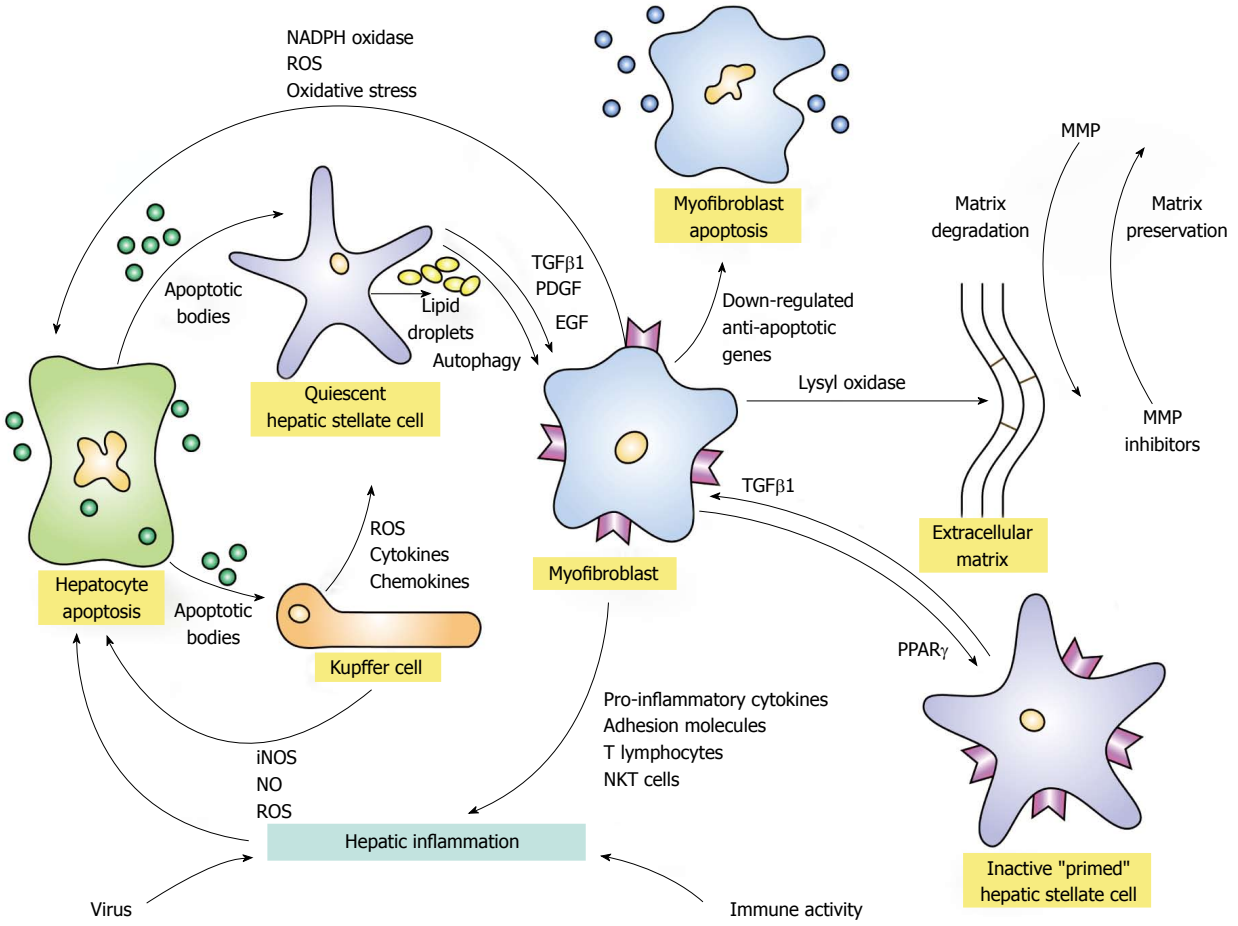


**Figure 1 Activation and de-activation pathways of hepatic fibrosis.** Hepatic inflammation triggered by virus infection or immune-mediated mechanisms can initiate fibrogenesis by inducing apoptosis of hepatocytes. The released apoptotic bodies can activate quiescent hepatic stellate cells and transform them into myofibroblasts under the mediation of transforming growth factor beta 1 (TGFβ1), platelet-derived growth factor (PDGF), and endothelial growth factor (EGF). The activation process is fueled by the release of lipid droplets (autophagy). The apoptotic bodies from the damaged hepatocytes can also stimulate Kupffer cells to generate reactive oxygen species (ROS) and nitric oxide (NO) by inducible nitric oxide synthase (iNOS). The ROS in turn can enhance hepatocyte apoptosis and continue the activation of hepatic stellate cells. Kupffer cells can also release ROS, cytokines and chemokines that contribute to the transformation of quiescent hepatic stellate cells to myofibroblasts. The myofibroblasts can also generate ROS and increase oxidative stress on hepatocytes by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway and promote hepatic inflammation by enhancing the expression of pro-inflammatory cytokines, adhesion molecules, activated T lymphocytes, and natural killer T (NKT) cells. The myofibroblasts generate the extracellular matrix which can be cross-linked by lysyl oxidases and rendered resistant to degradation by metalloproteinases (MMP). The reversibility of the extracellular matrix depends in part on the cross-linkage of collagen fibrils and the counterbalance of activities between MMP and MMP inhibitors. Myofibroblast activity can be attenuated by the down-regulation of anti-apoptotic genes which then favor myofibroblast apoptosis and the expression of the peroxisome proliferator-activated receptor-gamma (PPARγ) gene which may contribute to hepatic stellate cell inactivation. Inactivated stellate cells are different from quiescent hepatic stellate cells in that they are "primed" to have a low threshold for reactivation by TGFβ1.

on signaling pathways that influence the apoptosis of myofibroblasts, inactivation of hepatic stellate cells, and degradation of extracellular matrix (Figure 1). Myofibroblast apoptosis has been associated with the down-regulation of anti-apoptotic genes[44,45]; the peroxisome proliferator-activated receptor-gamma (*PPARγ*) gene has been associated with sustained quiescence of hepatic stellate cells and possibly their inactivation[45,54,55]; and metalloproteinases have degradative and fibrogenic properties that are counterbalanced[23,56,57]. Autophagy connotes the lysomal degradation of non-vital or dysfunctional cellular components, and these products can provide energy sources that maintain cell survival[58]. Hepatic stellate cells release lipid droplets (retinyl esters and triglycerides) during their activation, and this manifestation of autophagy may provide the energy necessary for their transition to myofibroblasts[59,60]. Clarification and potentiation of the signaling pathways that modulate these various actions can have therapeutic relevance.

Hepatic inflammation initiates fibrogenesis by promoting hepatocyte necrosis and apoptosis, sustains fibrogenesis by activating hepatic stellate cells and Kupffer cells, and maintains itself by the actions of pro-inflammatory cytokines and chemokines that influence the trafficking of inflammatory and immune cells within the liver[1,2] (Figure 1). The deposition of extracellular fibrillar collagen is the net result of these diverse and counter-regulated actions, and the degradation of this matrix becomes more formidable after cross-linkage of the collagen fibrils[61]. Diverse interactive and counter-regulatory mechanisms strive to maintain homeostasis, and current anti-inflammatory and immunosuppressive regi-

**Table 1 Anti-fibrotic actions and clinical outcomes of the conventional drug regimens**

| Conventional therapies | Possible anti-fibrotic actions | Clinical outcomes |
|---|---|---|
| Anti-viral agents | Prevent viral activation of HSC[72,74] | No fibrosis at 96 wk in 68%[7] |
| | Limit viral induction of ROS[74] | Less fibrosis after SVR in 33%[9] |
| | Reduce HSC proliferation[72] | Fibrosis stable in non-responders[6,9] |
| | Decrease pro-inflammatory signals[74] | Reversal of cirrhosis possible[10] |
| | Reduce TGFβ1 and procollagen[72-74] | Fewer complications of cirrhosis[10] |
| | Decrease lymphocyte recruitment[75] | Better transplant-free survival[10] |
| Corticosteroids | Inhibit NF-κB by stimulating IκB[85] | Less fibrosis in 57%[11] |
| | Deplete pro-inflammatory factors[86] | Reversal of cirrhosis[12,13] |
| | Decrease adhesion molecules[84,87] | Less or stable fibrosis in 79%[14] |
| | Increase lymphocyte apoptosis[88] | Inflammation increases fibrosis[3,14] |
| | Reduce production of ROS[49] | Cirrhosis survival improved[11,18,165] |
| | Suppress metalloproteinase inhibitors[90] | |
| | Decrease TGFβ1 activity[91-93] | |
| | Impair activation of HSC[94] | |
| Cyclosporine (calcineurin inhibitors) | Reduce cytokines and growth factors[162] | Decreased hepatic fibrosis score[15] |
| | Decrease lymphocyte proliferation[97,162] | Reduced inflammation score[15] |
| | Inhibit TGFβ and interleukin-4[164] | |
| Azathioprine | Deplete purine-based nucleotides[98-100] | Conjectural anti-fibrotic effects[97] |
| | Impair lymphocyte proliferation[98] | Used mainly with steroids[97,109] |
| | Increase lymphocyte apoptosis[105,106] | Possibly protective after relapse[110] |
| | Deplete NK cells[107] | |
| | Suppress pro-inflammatory genes[108] | |
| Mycophenolate mofetil | Inhibit lymphocyte proliferation[112,113] | Unproven anti-fibrotic effects[97] |
| | Increase lymphocyte apoptosis[112,113] | |
| | Decrease adhesion molecules[112,113] | |
| | Inhibit fibroblast proliferation[114] | |
| | Reduce iNOS production[112,113] | |
| Ursodeoxycholic acid | Limit apoptosis of hepatocytes[138] | Varied anti-fibrotic effects[118,120,125] |
| | Decrease oxidative stress[139] | Slows fibrosis progression[126] |
| | Reduce TGFβ1 signaling in HSC[140] | |

BA: Bile acids; HSC: Hepatic stellate cells; IκB: Inhibitor of nuclear factor kappa B; NF-κB: Nuclear factor kappa B; NK: Natural killer; ROS: Reactive oxygen species; SVR: Sustained virological response; TGFβ1: Transforming growth factor beta 1.

mens for the chronic inflammatory liver diseases have an important, but imprecise, role in supporting this effort. Interventions that target site-specific molecular pathways implicated in the fibrotic process promise to bolster these regimens[22-24].

## ANTI-FIBROTIC ACTIONS OF THE CONVENTIONAL DRUG REGIMENS

Anti-viral and immunosuppressive regimens have been the mainstays of therapy for the chronic inflammatory liver diseases, and the prevention or reversal of hepatic fibrosis has not been their primary objective. The dynamic nature of hepatic fibrosis had not been fully appreciated at the time of their introduction[62,63]; assessments of changes in hepatic fibrosis were hampered by sampling error and observer variation in the interpretation of liver tissue specimens[64-68]; and biomarkers more closely reflected liver inflammation than collagen deposition[69,70]. Subsequent investigations indicated that fibrosis could decrease during anti-viral therapy for chronic hepatitis C[4,9,10,71] and corticosteroid treatment for autoimmune hepatitis[11,12,14,15]. Furthermore, isolated clinical studies suggested that cirrhosis could disappear during treatment[13,14]. These experiences implicated hepatic inflammation as an important driver of liver fibrosis, and they indicated that the prevention or reversal of hepatic fibrosis was an appropriate goal of the conventional treatments for chronic inflammatory liver disease[3,14].

### *Anti-viral therapies*

Treatments that eliminate the hepatitis C virus (HCV) can impair virus-induced inflammatory, immune-mediated, and fibrogenic responses (Table 1). The hepatitis C virus can activate hepatic stellate cells[72,73], and viral proteins can increase oxidative stress within hepatocytes[1,23,74] and promote the release of pro-inflammatory chemokines[75]. The introduction of non-structural proteins of HCV into hepatic stellate cells *via* an adenovirus vector can increase the proliferation of these cells, stimulate chemokine secretion, and enhance expression of adhesion molecules[74]. Incubation of activated human hepatic stellate cells with recombinant HCV proteins increases the production of reactive oxygen species[1,23,74], and HCV proteins also stimulate the secretion of TGFβ1 and the production of pro-collagen in cultured rat hepatic stellate cells[74].

In patients with chronic hepatitis C, the chemokines, CCL21 and CCR7, are expressed within liver tissue, and CCL21 is concentrated around portal tracts and lymphoid nodules[75]. $CD8^+$ T lymphocytes isolated from the liver of these patients are more commonly positive for

CCR7 than cells from controls, and cultured hepatic stellate cells produce CCR7 and react to CCL21 by triggering pro-inflammatory signaling pathways that include NF-κB[75]. In this fashion, HCV can influence the intrahepatic cytokine milieu and support inflammatory and immune-mediated responses that favor fibrogenesis.

Hepatic steatosis is present in as many as 70% of patients with chronic hepatitis C[76-78]. In those patients infected with HCV genotype non-3, the frequencies of steatosis and fibrosis are higher in patients with oxidative stress[78]. Hepatic steatosis is independently associated with oxidative stress, and hepatic steatosis and the histological activity score are independent predictors of hepatic fibrosis[78]. These findings suggest that complex inter-dependent pathogenic pathways involving liver inflammation, oxidative stress, hepatic steatosis, and hepatic fibrosis are interwoven in some patients with chronic hepatitis C. Elimination of the viral agent might be the key to interrupting this fibrogenic process.

Anti-viral therapy may be anti-fibrotic because HCV infection promotes hepatic inflammation, immune-mediated responses, and signaling pathways that can enhance fibrogenesis (Table 1). HCV may also directly activate hepatic stellate cells without mediators of inflammation, and this possibility suggests a basis for the observed discrepancy between inflammatory activity and progressive hepatic fibrosis in some HCV-infected patients[72]. Other viral agents are not as well studied as triggers for fibrogenesis, but the association should be broadly accepted until proven otherwise. Elimination rather than attenuation of the viral infection should be the goal of treatment[79].

### Corticosteroids

Immune-mediated liver diseases are consequences of cell-mediated and antibody-dependent mechanisms that are directed against self-antigens[80,81]. Autoimmune hepatitis lacks an etiological trigger that can be precisely targeted, and its treatment is directed in a non-selective fashion at putative inflammatory and immune-mediated mechanisms of tissue injury that can in turn promote hepatic fibrosis[82] (Table 1). Prednisolone is the active metabolite of prednisone, and it binds to a glucocorticoid receptor within the cytosol[82,83]. This complex is then translocated to the nucleus where it interacts with glucocorticoid-responsive genes and inhibits the production of pro-inflammatory cytokines[82,84].

Prednisolone also has an intracytoplasmic effect on the activity of NF-κB by stimulating the production of its inhibitor (IκB)[82,85]. Nuclear factors essential for the transcription of cytokines are depleted, and pro-inflammatory and immune-stimulatory actions are suppressed[82,86]. These actions are augmented by a prednisolone-induced reduction in the expression of adhesion molecules necessary for the targeting of inflammatory and immune cells[84,85,87]. Furthermore, prednisolone enhances the apoptosis of lymphocytes, and it can thereby interrupt an injurious immune-response[88].

Prednisolone also has broad anti-fibrotic actions (Table 1). By reducing hepatic inflammation, the signaling pathways that trigger the production of reactive oxygen species may be less active[49]. Metalloproteinase inhibitors, which are stimulated by hepatic inflammation, may be less provoked, and the degradation of fibrillar collagens by unopposed metalloproteinases may proceed more freely[89,90]. The expression of TGFβ1 may also be reduced by a glucocorticoid-responsive element in the human *TGFβ1* gene promoter[91]. Furthermore, the activation and binding characteristics of TGFβ1 may be impaired by corticosteroids[92,93]. These actions may in turn reduce the transformation of hepatic stellate cells into myofibroblasts[94].

The net effect of these corticosteroid actions on the inflammatory and immune-mediated responses in autoimmune hepatitis is to limit tissue damage, reduce the signals for fibrogenesis, and restore homeostatic mechanisms that control the extracellular matrix. The multiplicity and diversity of corticosteroid actions[82,87] and the complexity and interconnectivity of the signaling pathways of fibrogenesis[1,2] limit the efficacy and consistency of corticosteroids as anti-fibrotic agents[23]. Cirrhosis is still a common consequence of autoimmune hepatitis[18,95], and corticosteroids have had variable effects on fibrogenesis in animal models[96].

### Azathioprine

Azathioprine is a purine antagonist that has anti-proliferative, pro-apoptotic, and anti-inflammatory actions that are complementary to the actions of prednisone and prednisolone, and these actions may in turn strengthen the anti-fibrotic actions of the corticosteroids[97] (Table 1). The 6-thioguanine nucleotides are the active metabolites of azathioprine, and they can impair the synthesis of purine-based nucleotides essential in the creation of new DNA and the proliferation of activated lymphocytes[98-102]. Intracellular signal transduction can also be blocked by the generation of 6-thioguanine triphosphate which in turn dampens immune cell proliferation[103]. Furthermore, the azathioprine-generated 6-thioguanine triphosphate can interrupt a dephosphorylation pathway necessary for the activation of T lymphocytes by antigen presenting cells[104]. These anti-proliferative actions can be complemented by pro-apoptotic and anti-inflammatory actions that may also impact on the signals for fibrogenesis.

Genes that regulate the expression of anti-apoptotic factors are inhibited by 6-thioguanine triphosphate, and the survival of the activated T and B lymphocytes that mediate liver injury may be shortened[105,106]. Natural killer cells that can contribute to an antibody-dependent cell-mediated liver injury may also be depleted[107]. These actions can reduce immune-mediated liver injury and secondarily, the inflammatory response to tissue damage. The 6-thioguanine nucleotides can also directly impair the inflammatory response by dampening the expression of pro-inflammatory genes[108].

The anti-fibrotic actions of azathioprine are conjectural and based on the putative actions of its active

metabolites[97] and its association with the clinical findings of reduced fibrosis in patients with corticosteroid-treated autoimmune hepatitis[14]. The preferred treatment of autoimmune hepatitis is prednisone or prednisolone in combination with azathioprine, and the anti-fibrotic contributions of azathioprine to the clinical experiences with corticosteroids can only be surmised[109]. Azathioprine (2 mg/kg daily) has been used as a long-term maintenance therapy in patients with autoimmune hepatitis who have relapsed after corticosteroid withdrawal, but its anti-fibrotic effects during such treatment have not been studied[110]. The stable quiescence of the disease during maintenance therapy with azathioprine suggests that the drug may prevent progressive fibrosis by preventing exacerbations of inflammatory activity[110,111].

### *Mycophenolate mofetil*

Mycophenolate mofetil is a next generation purine antagonist that has a different metabolic pathway than azathioprine but similar anti-proliferative and anti-inflammatory actions[97,112,113] (Table 1). The synthesis of purine-based nucleotides is impaired by mycophenolic acid, which is the active metabolite of the drug, and cell proliferation is reduced by reversible, non-competitive inhibition of inosine monophosphate dehydrogenase, the enzyme necessary for conversion of inosine monophosphate to guanosine monophosphate. Deficiencies in guanosine monophosphate can in turn dampen cell-mediated immune responses and antibody production[97,112,113]. Furthermore, mycophenolic acid can induce apoptosis of activated lymphocytes, suppress the expression of adhesion molecules, decrease the proliferation of fibroblasts, and impair the production of iNOS in macrophages[97,112-114]. By these mechanisms, mycophenolate mofetil can limit the survival of activated lymphocytes, decrease inflammatory activity, and reduce tissue damage mediated through nitric oxide production. The theoretical net effects of these actions would be to reduce tissue damage and fibrogenesis while favoring fibrinolysis by de-repressing metalloproteinases[114]. As with azathioprine, the anti-fibrotic effects of mycophenolate mofetil are unproven and not the primary objectives of treatment with this agent[97].

### *Ursodeoxycholic acid*

Ursodeoxycholic acid alone or in combination with corticosteroids has been an effective frontline therapy for autoimmune hepatitis in Japan[115-117] (Table 1). In other countries, it has been used mainly in diverse cholestatic liver diseases as the sole drug[118-120] or in syndromes with mixed features of autoimmune hepatitis and cholestasis ("overlap syndromes") in conjunction with corticosteroids[121-123]. Unlike the drugs used for chronic viral hepatitis or autoimmune hepatitis, the principal actions of ursodeoxycholic acid are not directed at reducing liver inflammation by either eliminating an etiological agent or interrupting immune-mediated pathways[124].

Experiences reporting stabilization of histological stage in treated patients with primary biliary cirrhosis (PBC)[120] have been countered by experiences reporting no or uncertain effects of the drug on hepatic fibrosis[118,125]. Treatment of PBC with ursodeoxycholic acid has been associated with a five-fold slower rate of progression from early stage to advanced stage disease compared to untreated patients[126], and the drug probably has an anti-fibrotic effect that is manifested by the slower progression of fibrosis. The impact of ursodeoxycholic acid on hepatic fibrosis takes years to recognize, and the treatment has not been associated with regression[126].

Importantly, hepatic inflammation, manifested as lymphocytic piecemeal necrosis, is an independent predictor of progressive hepatic fibrosis in PBC[127,128], and patients with PBC and inflammatory manifestations that resemble those of autoimmune hepatitis respond poorly to PBC-directed therapies[129]. They have higher frequencies of esophageal varices, gastrointestinal bleeding, ascites, and death from liver failure or requirement for liver transplantation than patients with classical PBC[129,130], and the presence of these inflammatory manifestations has justified treatment regimens that combine corticosteroids with ursodeoxycholic acid[121,131,132]. In PBC as in chronic hepatitis C and autoimmune hepatitis, hepatic inflammation is a driver of hepatic fibrosis.

Ursodeoxycholic acid has cytoprotective, bile stimulatory, and anti-apoptotic properties in chronic cholestatic liver disease, and its anti-inflammatory or anti-fibrotic effects are consequences of these three basic properties[124,133]. Phospholipids in mixed micelles protect cholangiocytic membranes against damage by hydrophobic bile acids[134], and ursodeoxycholic acid can modulate the composition of micelles to favor the phospholipid component[135,136]. The cytoprotective actions by this hydrophilic bile acid can in turn reduce or prevent cholangiocyte injury, portal inflammation, and the generation of fibrogenic stimuli. Ursodeoxycholic acid also stimulates biliary secretion of potentially toxic hydrophobic bile acids[137], and it can thereby protect against the apoptosis of liver cells whose death receptors would otherwise be directly stimulated by increasing intracellular concentrations of the toxic bile acids[138].

Ursodeoxycholic acid has had anti-fibrotic effects in a bile duct-ligated animal model[139], and it has been found to reduce the expressions of TGFβ1, TGF type 1 receptor and other components of the signaling pathway involved in the activation of cultured rat hepatic stellate cells[140]. Furthermore, ursodeoxycholic acid may reduce fibrogenesis through a pathway involved in the synthesis of glutathione which in turn may decrease oxidative stress and the activation of hepatic stellate cells[139]. These properties have had limited effects on hepatic fibrosis in humans with chronic cholestatic liver disease[118,120,125,126], and norursodeoxycholic acid, which is a homologue of ursodeoxycholic acid, may have more potent anti-inflammatory and anti-fibrotic actions[134,141].

Norursodeoxycholic acid has a shortened side chain which distinguishes it from ursodeoxycholic acid, and it is resistant to conjugation (amidation) with taurine or gly-

cine[141-143]. The unconjugated state and its shortened side chain renders the molecule more easily reabsorbed from bile and more rapidly re-secreted by hepatocytes[141,144]. This shunting within the enterohepatic circulation is associated with a hypercholeresis and decreased cholangitis and fibrosis in a murine model of primary sclerosing cholangitis[141]. Biliary fibrosis in murine models of chronic cholestatic liver disease correlate with the amount of ductular reaction[145-147], and norursodeoxycholic acid may attenuate this trigger for fibrosis by reducing ductular proliferation[141]. Clinical trials are needed to determine the efficacy and safety of this intervention.

## EFFICACY OF CURRENT TREATMENTS IN PREVENTING OR REVERSING HEPATIC FIBROSIS

The ability of conventional therapies to prevent or reverse hepatic fibrosis has been difficult to assess reliably because treatment regimens and follow-up schedules have varied and the methods for evaluating changes in hepatic fibrosis have been flawed. The sampling variations and interpretative inconsistencies of liver tissue examinations[23,64,66,148,149] have stimulated the quest for biomarkers[128,150-152] and imaging tests of hepatic fibrosis[151,153,154]. Liver tissue examination by needle biopsy, however, has remained the standard assessment of liver fibrosis as other modalities have been inaccurate, costly, or premature[152]. Furthermore, confidence in the liver tissue examination has been improved by the use of codified evaluation protocols for histological interpretation[155,156].

In chronic hepatitis C, the METAVIR scoring system developed by the French Cooperative Study Group has been the preferred scoring system[65,68,155], and in autoimmune hepatitis, the Ishak scoring system[3,14,156], which is a refinement of the earlier Knodell scoring system[157], has been preferred. Both systems are subject to sampling error and inter- and intra-observer variations[67,68]; each system can underestimate the grade and stage of the liver disease in small tissue samples[158]; and each system can anticipate a maximum staging accuracy of only 75% in tissue specimens that are $\geqslant$ 25 mm in length[68,148,159].

Importantly, the general availability of the needle biopsy assessment, the opportunity to acquire additional histological information about the disease and its response to treatment[148,160], the high concordance of the histological interpretations among pathologists (83%-84%)[67,68], and the strong association of the tissue findings with clinical outcomes[161] have counterbalanced the deficiencies intrinsic to the needle biopsy procedure. As a result, needle biopsy of liver tissue remains the principal basis for understanding the dynamics of hepatic fibrosis in patients with chronic hepatitis.

### *Anti-viral therapy and the prevention or reversal of hepatic fibrosis*

Pooled data from three randomized clinical trials involving 1509 patients with chronic hepatitis C demonstrated that patients who were treated with interferon alfa-2b in combination with ribavirin or interferon alfa-2b in combination with placebo for 48 wk had little or no hepatic fibrosis by METAVIR criteria at 96 wk more commonly than patients receiving the same regimens for 24 wk (68% *vs* 42% and 64% *vs* 24%, respectively)[7] (Table 1). The frequency of a sustained virological response, the duration of anti-viral therapy, and the histological stage of fibrosis prior to treatment were associated with the ability to prevent progressive hepatic fibrosis[7]. The efficacy of this treatment in limiting fibrosis was 68%.

Similar findings were reported in another study in which 99 patients with chronic hepatitis C were treated with interferon and ribavirin and 64 patients were treated with interferon alone[9] (Table 1). Progression of hepatic fibrosis, as assessed by changes in the METAVIR score and a semi-quantitative fibrosis score, was slowed more commonly in patients with advanced hepatic fibrosis who achieved a sustained virological response than in the non-responders[9]. Patients with cirrhosis at presentation who achieved a sustained virological response decreased their fibrosis score more commonly than non-responders (33% *vs* 9%), albeit fibrosis scores did not decrease by more than 2 points in any patient with cirrhosis. Importantly, the non-responders had no progression of hepatic fibrosis after 12 mo of therapy, and the anti-viral treatment may have had a protective effect even in the absence of a virological response[9], as had been reported in an earlier study[6].

The anti-fibrotic effects of anti-viral therapy in patients with chronic hepatitis C and cirrhosis have also been associated with fewer liver-related morbidities and better survival than in patients with unimproved hepatic fibrosis (Table 1). Of 96 patients with chronic hepatitis C and cirrhosis who received anti-viral therapy, 18 patients (19%) improved from stage 4 to stage 2 by the METAVIR scoring system after a median follow-up of 118 mo. Ten patients improved to stage 2; 7 patients improved to stage 1, and one patient improved to stage 0[10]. Improvements in the stage of fibrosis were associated with a reduction in the incidence of cirrhosis-related complications (ascites, hepatic encephalopathy, variceal bleeding, bacterial peritonitis, hepatocellular carcinoma, and liver transplantation) from 4 per 100 patient-years in individuals without regression of fibrosis to 0 per 100 patient-years in individuals with regression of fibrosis. Furthermore, the frequency of transplant-free survival at 10 years was higher in the patients in whom the fibrosis regressed (100% *vs* 74%)[10].

The composite experiences with anti-viral therapy in chronic hepatitis C confirm an anti-fibrotic effect, especially after sustained clearance of the virus, but the results are unpredictable, incomplete, and most often of low magnitude. Nevertheless, improvements in the fibrosis score have been associated with less morbidity and better survival in patients with chronic hepatitis C.

### *Immunosuppressive therapy in the prevention or reversal of hepatic fibrosis*

Corticosteroid therapy has improved hepatic fibrosis scores by a semi-quantitative scoring system in 57% of patients with autoimmune hepatitis who underwent paired needle biopsy examinations during a median follow-up of 49 mo, and the 10-year survival of all patients was similar to that of matched controls (90% *vs* 92%)[11] (Table 1). Another study indicated the loss of fibrosis and the reversal of cirrhosis (improvement in the median fibrosis score from 3.3 to 0.8 by Knodell scoring criteria) in 8 patients with autoimmune hepatitis and cirrhosis who responded to corticosteroid therapy[12]. Skepticism about the reversal of cirrhosis in needle biopsy specimens was somewhat allayed by the disappearance of cirrhosis in paired liver samples obtained by wedge biopsy in one treated patient after 14 years[13].

A larger study involving 325 liver specimens obtained by needle biopsy from 87 corticosteroid-treated patients with autoimmune hepatitis showed a reduction in the Ishak fibrosis score from 3.4 to 2.6 during a mean observation period of 63 mo[14]. Fibrosis scores improved in 53% of patients during a mean interval of 57 mo, and they did not worsen in 26% during a mean observation interval of 62 mo. In this study, corticosteroid treatment improved fibrosis or prevented its progression in 79% of patients, and the fibrosis score improved more commonly in individuals with reduced scores that reflected histological inflammation (61% *vs* 32%)[14].

The calcineurin inhibitors (cyclosporine and tacrolimus) can impair the activation of nuclear factors necessary for the transcription of cytokines and growth factors important in the proliferation of lymphocytes[97,162,163] (Table 1). These anti-proliferative actions can in turn reduce immune-mediated tissue injury and the recruitment of inflammatory cells to the sites of damage. Reduced production of TGFβ and interleukin-2 can have direct anti-fibrotic effects[164]. In 19 patients with autoimmune hepatitis who were treated with either cyclosporine (7 patients) or prednisolone (12 patients), mean fibrosis scores by the Ishak scoring system decreased from 4.5 to 2.2 during a mean interval of 3.4 years[15]. Reductions in the fibrosis scores were associated mainly with the use of cyclosporine and the duration of treatment, and the use of cyclosporine was the principal anti-fibrotic factor by logistic regression analysis (albeit the sample size was small and the confidence interval wide)[15]. Patients in whom fibrosis scores improved also had greater reductions in the scores for liver inflammation, and these findings reconfirmed the association between liver fibrosis and hepatic inflammation.

The composite experiences with immunosuppressive therapy in autoimmune hepatitis, including regimens based on the administration of cyclosporine, have indicated an anti-fibrotic effect in 57%-79%, and this improvement had been associated with reductions in hepatic inflammation. Reversal of cirrhosis is possible, but a limited reduction in the fibrosis score is more common.

## PROMISING SITE-SPECIFIC ANTI-FIBROTIC AGENTS IN CHRONIC LIVER DISEASE

Conventional treatments for chronic liver disease can improve hepatic fibrosis scores and increase survival, but these improvements are unpredictable, slow, and typically small. Cirrhosis in autoimmune hepatitis is uncommon during the first year of treatment (7%), but its occurrence increases to 39% at two years and 59% at 3 years if inflammatory activity continues[165]. The failure to induce resolution of hepatic inflammation within 36 mo is associated with higher frequencies of cirrhosis (54% *vs* 18%) and need for liver transplantation (15% *vs* 2%) than resolutions that occur within 12 mo[166]. In patients satisfying criteria for remission, the mean annual incidence of cirrhosis is 2.6%[165], and the risk of cirrhosis in autoimmune hepatitis persists indefinitely as a consequence of unsuspected residual or recurrent mild chronic inflammation[167,168].

The development of cirrhosis is also slow in chronic hepatitis C, but there is greater individual variability in this propensity than in autoimmune hepatitis depending on the age at the time of infection, daily alcohol consumption, and gender[169]. The median duration from infection to cirrhosis in untreated patients is 30 years, ranging from 13 years in men infected after the age of 40 years to 42 years in non-alcoholic women. Importantly, 31% of patients never develop cirrhosis or remain free of cirrhosis for at least 50 years[169]. These individual variations in the time to cirrhosis make assessments of the anti-fibrotic actions of anti-viral therapy difficult, but delays in clearing the virus or tolerating the medication probably contribute to disease progression[23].

Anti-fibrotic therapies have the potential to protect the liver during the protracted process of suppressing liver inflammation in autoimmune hepatitis and eliminating the etiological agent in chronic viral hepatitis (Table 2). Most anti-fibrotic therapies have theoretical value, limited laboratory evidence, and minimal or no human experience[22-24]. The most promising anti-fibrotic therapies that have been evaluated in humans with chronic liver disease have been the anti-oxidants[22,23]. The angiotensin inhibitor (losartin) has also had success in a limited non-randomized study[170]. Human trials of interventions that disrupt pro-inflammatory cytokine pathways mediated by tumor necrosis factor-α (infliximab[171-174], etanercept[175,176], and pentoxyphylline[177]), neurochemicals that block the fibrogenic activity of myofibroblasts (cannabinoid antagonists)[23,178], compounds that enhance the expression of nuclear receptors within hepatic stellate cells and preserve their quiescence (farglitazar)[179], and drugs that inhibit fibrogenesis by inactivating hepatic stellate cells, impairing TGFβ1 secretion, and protecting liver cells by increasing glutathione production and reducing oxidative stress (oltipraz)[180-182] have been ineffective or toxic.

**Table 2 Promising anti-fibrotic agents in chronic liver disease**

| Anti-fibrotic agent | Possible anti-fibrotic actions | Clinical experiences |
|---|---|---|
| Anti-oxidants | | |
| *N*-acetylcysteine | Inhibits NF-κB activity[195]<br>Limits pro-inflammatory genes[195]<br>Reduces iNOS and NO activity[196]<br>Decreases hepatocyte apoptosis[195] | Reduced hepatic fibrosis and inflammation in NASH[191] |
| *S*-adenosyl-*L*- methionine (SAMe) | Increase mitochondrial glutathione[197]<br>Inhibit NF-κB activity[197]<br>Reduce ROS production[197]<br>Impair iNOS and NO production[197]<br>Limit HSC activation[197] | Decreased mortality and LT in alcoholic cirrhosis[190]<br>Hastened decline of viral load and increased early response in HCV non-responders[192] |
| Vitamin E | Reduce TGF-β in animals and humans[199,200]<br>Decrease oxidant stress on hepatocytes[198]<br>Limit collagen deposition[198] | Decreased hepatic fibrosis in NAFLD[201]<br>Prevented progressive hepatic fibrosis in NAFLD[202] |
| Angiotensin inhibitors | | |
| Losartin | Limit angiotensin Ⅱ production by HSC[208]<br>Decrease expression of pro-fibrotic genes[170]<br>Limit NADPH-oxidase and oxidative stress[170]<br>Reduce TGF-β and pro-collagen production[214]<br>Decrease extracellular matrix[210,212,213] | Small trial in chronic hepatitis C[170]<br>Impeded pro-fibrotic and NADPH oxidase genes[170]<br>Reduced oxidative stress[170]<br>Decreased inflammatory and fibrosis scores in 50%[170] |

HCV: Hepatitis C virus; HSC: Hepatic stellate cells; iNOS: Inducible nitric oxide synthase; LT: Liver transplantation; NADPH: Nicotinamide adenine dinucleotide phosphate; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; NF-κB: Nuclear factor kappa-light-chain enhancer of activated B cells; NO: Nitric oxide; ROS: Reactive oxygen species; TGFβ: Transforming growth factor beta.

### *Anti-oxidants*

Oxidative stress is present in 61% of patients with chronic hepatitis C irrespective of histological activity index, viral load or viral genotype as assessed by immunoglobulin G antibodies against lipid peroxidation-derived antigens (malondialdehyde adducts to human serum albumin)[78]. Immunohistochemical staining of liver tissue using monoclonal antibodies against mouse macrophage iNOS and nitrotyrosine, which reflects nitric oxide production during inflammation, has indicated oxidative stress in all specimens from patients with primary biliary cirrhosis (14 patients) and autoimmune hepatitis (10 patients)[49]. The frequency of oxidative stress in chronic liver disease and the deleterious effects of reactive oxygen species on hepatocytes have supported the use of anti-oxidants as supplemental therapies, and studies evaluating these agents in animal models[183-188], cell lines[183,189], and humans with diverse liver diseases[78,190-192] have strengthened this role. The ability of anti-oxidants to reduce liver inflammation and disease severity has also advanced their promise as anti-fibrotic agents[193,194].

*N*-acetylcysteine is a sulfhydril donor that inhibits the transcription activities of NF-κB, and it can reduce the expression of pro-inflammatory genes[195], modulate the expression of iNOS[183,196], and limit apoptosis by reducing nitric oxide production (Table 2)[195]. Therapy with *N*-acetylcysteine in combination with metformin for 12 mo has improved histological activity scores and reduced hepatic fibrosis in patients with non-alcoholic steatohepatitis[191].

SAMe can replenish mitochondria with glutathione, inhibit NF-κB activity, reduce the generation of reactive oxygen species, and limit hepatic stellate cell activation by impairing the production of iNOS and the hepatic synthesis of nitric oxide (Table 2)[197]. Therapy with SAMe has decreased mortality or the need for liver transplantation in patients with alcoholic cirrhosis from 29% to 12%, and it has improved their two-year survival[190]. Therapy with SAMe (1600 mg daily for 2 wk) has also hastened the decline in viral load and increased the frequency of an early virological response (53% *vs* 0%) in non-responders with chronic hepatitis C (genotype 1)[192].

Vitamin E is an anti-oxidant that protects against toxic liver injury in animals[198] and prevents hepatic fibrosis in animal models and humans with acute and chronic liver damage (Table 2). Vitamin E reduces the production of TGFβ[199,200], and it in turn impairs the activation of hepatic stellate cells[199]. It has already been shown to improve[201] or stabilize[202] hepatic fibrosis scores in non-alcoholic fatty liver disease. Folate, melatonin, taurine, and salsalate are other anti-oxidants that are candidates for study in chronic liver disease[203]. Initial interest in betaine, as a method of increasing hepatic SAMe levels and reducing hepatic steatosis[204], in alcoholic[205] and non-alcoholic liver disease[206] has waned after performance of a controlled clinical trial[207]. Anti-oxidants have not been used in autoimmune hepatitis, but the results of their use in patients with alcoholic cirrhosis, non-alcoholic steatohepatitis, and chronic hepatitis C compel their consideration.

### *Angiotensin inhibitors*

Components of the renin-angiotensin system are expressed in multiple organs, including the heart, kidney, gonads, pituitary, adrenal glands, and liver[208,209], and angiotensin Ⅱ, which is the principal product of this system, can be synthesized by activated hepatic stellate cells[208]. Locally produced angiotensin Ⅱ from myofibroblasts is involved in the healing response to tissue injury,

and it can induce the secretion of pro-inflammatory cytokines and the synthesis of extracellular matrix as well as inhibit collagen degradation[170,210-212]. The fibrogenic properties of angiotensin II are consequences of reactive oxygen species that are generated within hepatic stellate cells by NADPH oxidase, and interventions that disrupt the renin-angiotensin system reduce experimental hepatic fibrosis and oxidative stress[213,214].

Losartan, an angiotensin receptor antagonist, has been assessed as an anti-fibrotic agent in a small clinical trial (Table 2). Fourteen patients with chronic hepatitis C were treated for 18 mo with losartin (50 mg daily)[170]. Inflammatory activity and fibrosis stage by the METAVIR scoring system decreased in 7 patients, and the expression of profibrotic genes and genes affecting NADPH oxidase activity and oxidative stress were also reduced[170]. Viral load, serum liver tests, collagen content, and fibrosis stage were unchanged overall, but the encouraging results in 7 patients justified a recommendation for a randomized clinical trial[170].

## OVERVIEW

Hepatic fibrosis can be prevented or reversed by eliminating the etiologic agent or disrupting the pathogenic mechanisms of liver injury. Studies in animal models of bile duct ligation[215] and schistosomiasis[216] and clinical experiences in patients with chronic bile duct obstruction[217], hemochromatosis[218], Wilson disease[219], jejuno-ileal bypass[220], thalessemia[221], primary biliary cirrhosis[222], chronic viral hepatitis[4-10], and autoimmune hepatitis[11,12,14,15] attest to this possibility[223]. Hepatic inflammation is only one driver of hepatic fibrosis, but it is a injurious process that can be measured by laboratory and histological indices and targeted by conventional therapies[3].

Current management strategies for the chronic inflammatory liver diseases have not been optimized to prevent or reverse hepatic fibrosis, and their potential salutary effect on this response to tissue injury has often been underestimated, unrecognized, or ignored[18,165,224]. Treatments of chronic viral hepatitis and autoimmune hepatitis are typically protracted[225-229], and advanced fibrosis and cirrhosis commonly develop late in the clinical course[23,165,169]. In autoimmune hepatitis, cirrhosis can emerge years after the presentation[165,167,168].

Conventional treatment strategies must be modified to focus on the prevention of hepatic fibrosis, and these modifications must ensure rapid viral clearance[166,226] and quick complete suppression of liver inflammation[230-233]. Furthermore, the resolutions must be durable[111]. The achievement of these objectives require early identification of the slow- or non-responder, reliable assessment of the tissue response, individualized adjustments in the treatment regimens, and the early incorporation of supplemental anti-fibrotic interventions of proven efficacy. Indefinite continuous therapy may be required in some patients[234]. Furthermore, anti-fibrotic agents are feasible as adjunctive therapies, and the identification and characterization of the preferred agent may be disease-dependent[235]. Randomized clinical trials are warranted to assess these issues, and they are only possible through a collaborative network of clinical investigators that is supported by a societal commitment to fund these studies[236].

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

# Evaluation of renal function in patients with cirrhosis: Where are we now?

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## Abstract

In the clinical context of the patients with liver cirrhosis, accurate evaluation of the renal function is potentially crucial. Indeed, it can lead to early diagnosis of both acute kidney injury and chronic kidney disease and to reliable characterization of the renal status of the patient before performing a liver transplantation. Despite some limitations, the assay of serum creatinine (SCr) is universally used to estimate glomerular filtration rate (GFR) because of its wide availability, its simplicity and because it is inexpensive. Nevertheless, several reports show that the value of this assay to estimate GFR is strongly challenged in cirrhotic patients, especially in patients with liver failure and/or severely impaired renal function. This has led to seek new alternatives to estimate more reliably the GFR in these patients. Although the reference methods, based on the utilization of exogenous markers, allow measuring GFR and thereby constitute the "gold standard" to evaluate renal function, they are not feasible in routine clinical practice. Several studies have shown that a cystatin C (CysC) based formula perform better than the SCr-based estimates in cirrhotic patients and the estimation of GFR by these formulas could therefore lead to optimize the management of the patients. A new estimate based on CysC has been recently developed using a large number of patients and the first results regarding the evaluation of its performance are promising, making this new formula the best candidate for a reference estimate of the renal function in cirrhotic patients.



**Key words:** Cirrhosis; Glomerular filtration rate; Formula; Estimation; Agreement; Plasma creatinine; Cystatin C

**Core tip:** Cirrhotic patient management frequently requires evaluation of renal function. However, these patients present some specific disturbances that affect the serum creatinine value, making its use to estimate glomerular filtration rate unsuitable. To get a more appropriate evaluation of the glomerular filtration rate, other methods are available such as the use of exogenous markers or assaying cystatin C in the blood, which avoid the drawbacks of the serum creatinine. Recently, a convenient new cystatin C based formula was tested and showed correct performance in cirrhotic patients, even in case of liver failure and/or severely decrease renal function.

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## INTRODUCTION

Liver cirrhosis (LC) is a frequent disease with various causes and a severe prognosis. Thus, after a first episode

of decompensation, the 5-year mortality in the absence of liver transplantation (LT) is as high as 85%[1]. Renal impairment, whether acute or chronic, is a highly prevalent comorbid condition in cirrhotic patients, which is associated with a poor prognosis[2]. In this clinical context, acute kidney injury (AKI)[3] is frequent and often of functional origin (around 70%). However, AKI of other origin are not rare, mainly secondary to hepato-renal syndrome (HRS), drug nephrotoxicity or severe sepsis[4]. Chronic Kidney Disease (CKD) is not infrequent as well and can be of various origins (glomerulonephritis, diabetic nephropathy or hypertensive nephrosclerosis). Although several studies assessed the frequency of renal impairment in patients with cirrhosis, it is not always clear whether it was acute or chronic kidney disease. About the prevalence of CKD, several studies suggest a prevalence of CKD stage 3 or higher (*i.e.*, estimated Glomerular Filtration Rate (eGFR) < 60 mL/min per 1.73 $m^2$) between 20% and 40%. In a study including more than 1400 cirrhotic patients who underwent an evaluation of renal function by a reference method in pre LT clinical assessment, 11.3% had a GFR below 40 mL/min[5]. In our cohort of alcoholic cirrhotic patients, about 40% had a measured GFR below 60 mL/min per 1.73 $m^2$[6]. Finally, in the study by Ojo *et al*[7] a prevalence of 26.8 % of stage 3-5 CKD was found in patients who subsequently received a liver transplant between 1990 and 2000 in the United States [however, in this study, analysis was based on the eGFR instead of measured GFR (mGFR)]. Nevertheless, it is likely that some of these studies provide an underestimated prevalence of CKD in cirrhotic patients because they included only candidates for LT, whereas the CKD prevalence may be higher in patients contraindicated for receiving a LT. Moreover, it is not always known whether the renal impairment might have been (at least partly) acute in these studies. About the frequency of AKI in cirrhotic patients, some authors found that it could occur in 50% to more than 90% of patients in the perioperative period of LT and in 20% of hospitalized patients with LC[4].

The detrimental clinical impact of the existence of either CKD and/or AKI on the outcomes of cirrhotic patients has been highlighted by several studies. About the impact on mortality, a recent systematic review summarized results from 74 studies that assessed the effect of renal failure on early mortality in cirrhotic patients and found an increased risk of death with a pooled odds ratio of 7.6. Whether the renal failure was acute or chronic in some studies included in the systematic review was not clear but an increased risk of death was found in studies in which renal failure was defined as an acute renal failure and in those which renal failure was not clearly defined as chronic or acute (pooled odds ratio of 6.38 and 7.39 respectively). Although analysis in this study found significant heterogeneity (consequence of the heterogeneous definition of the renal failure used in some studies) the majority of the studies found an increased odds ratio, which strongly suggests a negative impact of impaired renal function, either acute or chronic, on the survival of cirrhotic patients[8]. In case of subsequent LT, the presence of prior CKD or the occurrence of AKI has also a negative impact on both survival and "renal" prognosis of the patients. Indeed, it is known that the occurrence of perioperative AKI or the existence of preLT CKD decreases the survival of liver transplant recipient[4]. Furthermore, pre-transplant CKD promotes the development of post-transplant CKD and/or is associated with increased risk of End-Stage Renal Disease (ESRD) requiring renal replacement therapy (RRT) during follow-up[4,7]. In summary, the cirrhotic patients may face clinical situations with increased risk of acute and/or chronic renal disease and the occurrence of renal disease is known to have strong prognostic implication. Therefore, it appears that accurate evaluation of renal function is important firstly to optimize the management of these patients and secondly to properly determine patients prognosis in order to prioritize access to LT. Taken together, the previously cited data suggest that the level of renal function is a parameter of crucial importance that should be determined (sometimes iteratively) in the clinical evaluation of cirrhotic patients in order to optimize their management.

## DIAGNOSIS OF AKI

According to the recent Kidney Disease Improving Global Outcomes (KDIGO) guidelines about AKI, the diagnosis of AKI should be based on Serum Creatinine (SCr) increase and urine output, whereas RIFLE criteria, which were former reference in the field and are still largely used, were additionally based on GFR decrease. Although SCr is the historical marker, cheap and widely available, it is just a marker of renal function and thus increases tardily after the beginning of injury[4]. New marker such as Neutrophil Gelatinase Associated Lipocalin (NGAL) is able to detect renal parenchymal damage before SCr increase and thus allows, theoretically, to initiate early treatment that might mitigate the severity of AKI. It is a crucial point to optimize management of cirrhotic patients with AKI because some authors found that the mortality is related to the severity of renal failure[8,9]. So far, some studies suggested an interest of using NGAL assay in cirrhotic patients in the diagnosis of renal dysfunction, In the study by Verna *et al*[10] the authors found the ability of elevated NGAL level to predict independently short-term mortality in cirrhotic patients. Moreover, Fagundes *et al*[11] showed that NGAL increase was useful to differentiate AKI due to acute tubular necrosis from CKD and HRS as well as to differentiate HRS from CKD. However, to our knowledge, no studies clearly showed a positive clinical impact of the NGAL use in the management of the cirrhotic patients and more studies are needed to ascertain the clinical interest of this new marker in this context.

## DIAGNOSIS OF CKD

According to the KDIGO clinical practice guidelines for

the evaluation and management of CKD, CKD is defined as the existence since at least 3 mo of abnormalities of kidney function and/or structure with implications for health. The persistence of GFR below 60 mL/min/1.73 $m^2$ is one of these abnormalities and in case of the existence of other criteria, calculation of GFR is requested to determine the stage of the CKD[12]. Therefore, calculation of GFR is a key element in detection and/or staging of CKD. So, we will focus on the methods that might allow evaluating reliably the renal function and on the studies that assessed the performance of these methods.

## METHODS OF EVALUATION OF THE RENAL FUNCTION

The GFR is the universally used index to quantify kidney function (with value given in mL/min). It can be measured by using a reference method of GFR measurement, or estimated, by using an endogenous marker (typically SCr) and different formulas (also called equations). In case of GFR measurement, the principle is to determine the body clearance of a substance with supposed exclusive renal elimination. The substance used is also supposed to be freely filtered and neither secreted nor reabsorbed along the renal tubule. In all probability, no extrarenal excretion of the substance occurs and it cannot be stored or be bound to plasma proteins: then it can be assumed that the plasmatic clearance is only due to renal clearance. Thus, the GFR can be inferred, at least theoretically, from the plasma disappearance of the substance. Considering the renal clearance of a marker occurs only through glomerular filtration, then the following relationship is satisfied: the amount that leaves the body per unit of time is strictly equal to the quantity of the same substance that appears in the urine per unit of time: [S]p X GFR = [S]u X Vu (with [S]p and [S]u respectively the plasma and urine concentration of the substance and Vu the volume of urine during a certain amount of time)[13].

Secondarily, a normalization on arbitrarily fixed body surface area (BSA) set to 1.73 $m^2$ is commonly done on the assumption that the GFR is positively correlated with the basal metabolism rate of individuals which is proportional to their stature[14]. Some authors have questioned this normalization[15] and standardization on other criteria (for example, the volume of total body water) has been proposed[16]. Nevertheless, the adjustment on the body surface remains widely used. The formula most commonly used to determine the BSA is the Dubois formula[17].

## REFERENCE METHODS: HOW TO MEASURE GFR?

These methods utilize exogenous markers, which should present several properties to be considered as "ideal markers". These properties include free filtration in the glomerulus without secretion nor reabsorption by the tubule, unable to bind to plasma proteins and with exclusive elimination by the kidneys. Moreover, the dosage of the compound must be accurate, inexpensive, and without interference with other plasma components. Finally, there should be no side effects for the patient[13]. The commonly used exogenous markers in clinical practice are inulin, iohexol and iothalamate. Several methods can be used to measure the GFR. The method originally proposed by Homer Smith is still one of the most frequently used. It is based on the continuous infusion of exogenous marker by varying the infusion rate until a stable plasma concentration is reached[13]. Urine collection over several time periods is then performed and the final GFR is the mean value of these measurements. Three samples are generally collected but up to five may be necessary. Although being a "gold standard" method, it has several drawbacks. It is time consuming, requires trained and experienced staff, the marker can be relatively expensive and the assay of inulin is sensitive to changes in glucose. Finally, the utilization of a bladder catheter may be required to exclude the impact of a problem of bladder voiding that can artificially reduce the GFR value[13]. Because of these drawbacks, other investigators have proposed simpler methods without urine collection. The proposed technique is to measure the infusion rate of the marker required to obtain a constant plasma concentration of the marker. Assuming that elimination is exclusively renal then the value of the infusion rate permits determination of the GFR value. Another increasingly used technique is to measure the disappearance of the marker in the plasma after a bolus infusion. This technique requires using a model of the behavior of the marker in the body to deduce the GFR value. Obviously, the reliability of the GFR measurement strongly relies on how realistic the model used is. Some investigators have demonstrated these bolus techniques allow appropriate assessment of the GFR by retrieving just two blood samples. In some studies just one assay was sufficient during the decrease phase of the marker concentration in the plasma. However, the single sample methods require careful timing of sample retrieval that must be chosen based on the expected level of renal function. Alternatively, the sample is taken according to the presence of certain clinical conditions: at least several hours after injection are required for normal renal function, whereas a sample after a longer period of time is needed when renal impairment is expected or if ascites is present[18]. Alternatively, radioactive (isotopic) markers can be used instead of usual marker: the most commonly used isotopic markers are the $^{125}$I-iothalamate, $^{51}$Cr-EDTA and $^{99}$Tc-iodohippuran[13]. In cirrhotic patients, some authors have reported a risk of overestimation of the GFR when alternative methods to urine collection are used, due to the possible existence of extra-renal clearance of the marker[19]. This seems to be true when the samples of plasma are taken too early after the bolus marker administration. Indeed, investigators have recently stressed the faster initial decrease and slower subsequent decrease in plasma marker concentration in patients with fluid overload[20]. In such cases, late measures might correct the overestimation by compensating for the faster initial decrease, but this remains to be confirmed. Therefore, the most reliable reference method appears to

be the "classic" Homer Smith method with the collection of urine and prior administration of an exogenous marker until reaching the equilibrium concentrations. However, as stated above, this is time consuming and necessitates trained staff.

## SERUM CREATININE

This endogenous marker of renal function is used universally as it is simple to measure, inexpensive and easily accessible. Initially, SCr was used for the assessment of renal function due to the assumption that its production remains broadly stable over time if the body weight was also stable. In addition, it was assumed that SCr production among gender-, weight- and age-matched patients was comparable. However, in patients with severe cirrhosis, daily creatinine production is decreased comparing with patients from the general population for two main reasons. Liver failure is responsible for decreased creatine production while some degree of malnutrition causes decreased conversion of creatine to creatinine. Therefore, the potential of SCr to be a reliable marker of renal function is strongly challenged in this clinical setting[21]. Additional difficulty when interpreting the value of SCr in cirrhotic patients comes from the interference, when using the Jaffe assay, of "non-creatinine" chromogens present in the plasma (typically bilirubin)[22]. Recently, Kuster *et al*[23] showed that comparing with an enzymatic assay, even a compensated Jaffe assay accounted for an average decrease of 6.14 μmol/L of the SCr in cirrhotic patients. This resulted in a median overestimation of GFR estimated by CKD-EPI formula and a reduced MELD score in patients with SCr > 1 mg/dL. Finally, it is known that there is a significant secretion of creatinine by the tubule in patients with decreased renal function, which increases when the CKD becomes more severe[24]. Several studies sought to determine the ability of SCr to estimate renal function and to detect CKD in cirrhotic patients, by using a reference method to measure GFR (Table 1). They showed that a large proportion of cirrhotic patients with moderate to large decrease in GFR had normal or just slightly increased SCr[25-28]. Moreover, some studies also found a non-significant correlation between 1/SCr or log SCr with GFR or poor performance of 1/SCr for detecting a decrease in GFR[26-27]. Apart from questioning the level of SCr that should be considered as really "normal" in cirrhotic patients, other previously cited factors contribute to jeopardizing the capacity of SCr as a reliable marker of the true GFR in cirrhotic patients. Therefore, what is the true clinical meaning of SCr in patients with severe cirrhosis? Assuming the absence of measurement error, SCr reflects a mix of clinical parameters including the degree of liver dysfunction, malnutrition and the patient GFR. Nevertheless, it was included in the MELD score, now widely used to prioritize patients in the access to LT, because of its (expected) capacity to serve as a proper marker of renal function. However, because of all the limitations previously cited, some authors have since highlighted the limitations of the use of SCr into the MELD score to properly classify the patients with the most severe cirrhosis[20,29-31]. It is well established that the MELD score penalizes patients that, in absence of any renal impairment, exhibit lower SCr, especially women[30,32]. In a study from our group in patients with severe alcoholic cirrhosis, we found lower SCr in women than in men, despite lower GFR in female patients[6]. Some studies have shown that replacing the SCr by eGFR or mGFR allowed more accurate classification of patients awaiting LT according to their risk of death[33,34]. This raises questions about the need to refine the MELD score in order to achieve a fairer assessment amongst cirrhotic patients awaiting LT.

## CREATININE CLEARANCE

It is a simple method to estimate GFR, based on the assumption that creatinine has the characteristics of a perfect renal marker. It requests the patients are able to collect accurately the urines from a 24 h period. Although very convenient, it has several limitations: mainly, the occurrence of tubular secretion of creatinine (which leads to overestimation of the GFR) and the possible inadequate urine collection by the patients, that is on a longer or shorter than 24h time period. Calculation of the eGFR requires normalization to BSA. Studies that tested the performance of this method showed a clear trend to overestimate mGFR by 4%-80%[25,27,35-37] (Table 1). In a meta-analysis including data from seven studies with 193 cirrhotic patients, Proulx *et al*[38] found a mean bias of +13 mL/min per 1.73 $m^2$ between GFR estimated by the Creatinine Clearance method (CrCl) and GFR measured by the inulin clearance. The authors also found that the bias tended to be higher in patients with lower GFR with a mean overestimation of 18% in patients with GFR > 60 mL/min per 1.73 $m^2$ and of 49% in patients with GFR < 60 mL/min per 1.73 $m^2$. The relationship between GFR level and overestimation could be explained by the secretion of creatinine by the tubule in patients with CKD. However, the importance of this overestimation does not seem to be related to the severity of cirrhosis. Some investigators have suggested that pharmacological inhibition of creatinine secretion by means of cimetidine could help to get more robust estimation of GFR with the CrCl[13,39]. However, limitations such as the effective level of tubular secretion inhibition that can be obtained with cimetidine remain. Cimetidine can have varying effects depending on several factors and the clinical safety of cimetidine administration is a matter of concern. To our knowledge there is no study that evaluated the performance of CrCl with cimetidine administration in cirrhotic patients. In conclusion, because of its limitations, the CrCl method is not largely used to estimate GFR in current clinical practice.

## SERUM CREATININE BASED FORMULA TO ESTIMATE GFR

They are probably the most widely used in current clini-

**Table 1 Summary of the results of the main studies which evaluated the performance of renal function markers and/or glomerular filtration rate estimates estimates comparatively to a reference method in patients with cirrhosis**

| Ref. | Number of patients | Reference method | Performance of the estimate(s) |
|---|---|---|---|
| Papadakis *et al*[25], 1987 | 23 (mGFR = 66) | Inulin | Difference between mean mGFR and ClCr and CG -24 and -52 mL/min respectively in group with decreased mGFR (+10 and +4 in patients with normal mGFR) |
| Caregaro *et al*[35], 1994 | 56 (mGFR = 86.7) | Inulin | Difference between mean mGFR and ClCr and CG -14.6 and -4.9 respectively. Mean overestimation was 51% and 40% respectively in patients with GFR < 80 |
| Roy *et al*[36], 1998 | 30 (mGFR = 30) | Inulin | Mean relative overestimation 80% with ClCr when moderate to severe CKD |
| Orlando *et al*[37], 1999 | 20 | Inulin | Mean relative overestimation of 4% and 23% respectively for ClCr and CG in Child C patients. Relative difference only +3% and -6% respectively in Child A patients |
| Woitas *et al*[26], 2000 | 44 (mGFR = 37) | Inulin | Sensitivity to detect GFR < 90, 85.7% and 28.5% respectively for elevated CysC and SCr |
| Demirtaş *et al*[27], 2001 | 26 (HRS) (mGFR = 33.5) | $^{99}$Tc-DTPA | Difference between mean mGFR and ClCr +7 |
| Orlando *et al*[28], 2002 | 36 (mGFR = 71.5) | Inulin | Mean overestimation was 75% and 30% respectively for CG and ClCr in patients with decreased GFR (14% and 9% in patients with normal GFR). Sensitivity to detect GFR < 72 were 73%, 23%, 53% and 86% respectively for elevated CysC and SCr, CG and ClCr |
| Gonwa *et al*[5], 2004 | 1447 (Pretransplant) (mGFR = 90.7) | $^{125}$I-Iothalamate | P30 were 60.8% and 66.7% for respectively CG and MDRD4. Difference between means mGFR and CG and MDRD4 +23.5 and +21.9 respectively |
| Pöge *et al*[41], 2006 | 44 (mGFR = 35.3) | Inulin | Mean absolute bias and P30 was 51.7/4.5%, 48.3/6.8%, 33.3/11.4% and 33.9/13.6% for respectively CG, MDRD4, Hoek and Larsson GFR formula |
| MacAulay *et al*[42] 2006 | 57 (mGFR = 83) | $^{99}$Tc-DTPA Iohexol | Mean difference between formula and mGFR was lower for MDRD6 comparing with CG (+3.5 *vs* +15.4). However, mean absolute difference was high and similar (23.4 *vs* 23.6) and poor precision was found with both eGFR (root mean square error 31.5 *vs* 30.5 for respectively MDRD6 and CG) |
| Francoz *et al*[31], 2010 | 157 (mGFR = 85) | Inulin | Mean absolute bias ± SD was 17 ± 32, 16 ± 29 and 8 ± 22 for CG, MDRD4 and CKD-EPI respectively. In patients with GFR < 70, CKD-EPI bias rose to 19 ± 20 |
| Rognant *et al*[6], 2010 | 148 (Alcoholic Cirrhosis) (mGFR = 77) | | Median absolute bias ± SD and P30 was 23 ± 23/33.3% and 22 ± 20/40% for CG and MDRD4 respectively |
| Kim *et al*[43], 2011 | 89 (normal SCr) (mGFR = 73) | $^{99}$Tc-DTPA | Difference between mean mGFR and ClCr, CG and MDRD6 was -14.4/+ 19.1 and -40.1 respectively. AUC of ROC to detect GFR < 60 was 0.721, 0.561, 0.463 and 0.659 for 1/CysC, ClCr, CG and MDRD6 respectively |
| Xirouchakis *et al*[47], 2011 | 74 (mGFR = 81.7) | $^{51}$Cr-EDTA | Concordance correlation coefficient was 0.61, 0.38 and 0.46 for respectively MDRD4, Larsson and Hoek estimates. P30 was 64% for MDRD4 and 68% for Hoek. |
| Gerhardt *et al*[48], 2011 | 44 (mGFR = 35.3) | Inulin | Median absolute bias and P30 was 40.1/6.8% and 42.5/6.8% for respectively MDRD175 and CKD-EPI |
| De Souza *et al*[49], 2013 | 202 (Pretransplant) (mGFR = 83) | Inulin | Concordance correlation coeffcient and P30 was 0.75/78.7, 0.56/42.6, 0.62/56.4, 0.8/83.2 and 0.82/78.2 for respectively Hoek, MDRD175, CKD-EPI, CKD-EPI CysC and mixed CKD-EPI formula |

Acronyms description can be found in the text. GFR: Glomerular filtration rate; HRS: Hepato-renal syndrome; CKD: Chronic kidney disease; CG: Cockcroft and Gault formula; SCr: Serum creatinine.

cal practice to assess the GFR because estimation can be obtained quickly and easily. The parameters of the population used to work out the main SCr based formulas are given in Table 2. This information is important to take into account to understand the poor global performance of these formulas in cirrhotic patients. Indeed, it appears unlikely that some cirrhotic patients were included in the populations used to elaborate these formulas.

Historically, the Cockcroft and Gault formula (CG) was the most popular before the MDRD formula was published in the early 2000s. It was developed in the early 70s using population data from 249 men. Furthermore, it is important to note that the reference method used to develop this formula was the CrCl method, which is not really a reference method[40]. This formula is not adjusted to the patient BSA and the adjustment has, theoretically, to be done afterwards (even if the relevance of this adjustment remains to be assessed in cirrhotic patients). Repeated testing of the CG formula in cirrhotic patients confirmed poor performance in most of the studies[5,6,25,28,35,37,41-43]. Similarly to the CrCl, the CG clearly tends to overestimate GFR, especially in some clinical contexts. According to major studies in the field, this overestimation may be between 5 and 51 mL/min, in some instances reaching 80% of the mGFR value. This overestimation seems to be more important for lower GFR and more severe cirrhosis as well[5,28,37]. Another point of concern is the impact of BSA normalization of the eGFR when evaluating CG performance. Not every study utilized normalized eGFR, which may have a confounding effect on the results. Intuitively, the overestimation in patients with large retention of ascites that are artificially overweight may decrease. Apart from our group[6], several other authors have underlined the limitations of the CG formula in the assessment of renal function in cirrhotic patients[18,29].

The MDRD formula was developed in 1999 in a large-sized North American population, which was more heterogeneous than the one used to derive the CG formula (Table 2). In addition, the authors utilized

**Table 2 Description of the characteristics of the studies used to develop the common glomerular filtration rate estimates**

| Name of the study | Number of patients | Country | Reference method | Marker(s) | Mean GFR | Comments |
|---|---|---|---|---|---|---|
| CG 1976 | 249 | Canada | 24 h ClCr | SCr | 30-130 | No normalization on BSA<br>Male patients only in the population of the study |
| MDRD 1999 | 1628 | United States | Renal clearance $^{125}$I-Iothalamate | SCr | 39.8 ± 21.2 | Characteristics of the study population:<br>Male 60%<br>Black patients 12%<br>> 55 yr 42%<br>Diabetic patients 6%<br>Re-expressed in 2007 to be used with IDMS traceable creatinine assay (MDRD 175) |
| CKD EPI (PCr) 2009 | 8254 | United States | Various (urinary clearance of exogenous markers) | SCr | 68 ± 40 | Characteristics of the study population:<br>Male 57%<br>Black patiens 32%<br>> 65 yr 13%<br>Diabetic patients 29% |
| Hoek 2003 | 123 | The Nederlands | Renal clearance $^{125}$I-Iothalamate | CysC | Median = 81 | Characteristics of the study population:<br>Male 48%<br>Median age 50 yr<br>Diabetic patients 24% |
| CKDEPI (Cys C and mixed PCr + CysC) 2012 | 5352 | United States | Various (urinary or plasma clearance of exogenous markers) | CysC alone and both CysC and SCr | 68 ± 39 | Characteristics of the study population:<br>Male 58%<br>Black patients 40%<br>Age > 65 yr 13%<br>Diabetic patients 32%<br>Patients with BMI > 30 31% |

Acronyms description can be found in the text. GFR: Glomerular filtration rate. CKD: Chronic kidney disease; CG: Cockcroft and Gault formula; BMI: Body mass index.

a measured GFR as the reference method and provided an eGFR normalized to BSA[44]. Initially, several MDRD formulas were developed, with the simplest or 4-variables MDRD including SCr, age, gender and ethnical origin. This formula rapidly became the most popular compared to the 6-variables MDRD formula, which additionally requires blood urea nitrogen and serum albumin concentration to estimate GFR[45]. In 2007, the formula with 4 variables (MDRD4) has been re-expressed for SCr measured with assay traceable to the IDMS reference assay. This formula is also known as MDRD 175, which refers to the first multiplicative factor of the equation[46]. The performance of these formulas has been tested several times in cirrhotic patients[5,6,34,41,42,43,47-49]. The studies have shown, as for the CG, a clear tendency to overestimate mGFR with a bias between 15 and 48 mL/min per 1.73 $m^2$ depending on the average GFR of the patients included in the studies. As for the CG formula, the level of the bias is inversely proportional to the level of the mGFR. Agreement between MDRD eGFR and measured GFR assessed *via* the accuracy 30% (which is the proportion of patients with eGFR between mGFR minus 30% and mGFR plus 30% and is also called P30) is poor. Indeed P30 was between 6.8 % and 42.6 % depending on the study. Importantly, MDRD formulas did not seem to perform better than CG formula, whereas, our recent study suggested that the performance of MDRD6 is possibly better than other SCr based formulas (but remained lower comparing with CysC based formulas). In 2009, the Levey group, which developed the MDRD formula, published a new formula for estimating GFR. It was based on the same parameters as the MDRD4 and used measurements of the GFR collected from more than 8000 patients[50]. The mean mGFR of the population was higher than for the MDRD formula (68 mL/min per 1.73 $m^2$). The main advantage of this new and more complicated formula named CKD-EPI, is the lower underestimation of the eGFR comparing with the MDRD for GFR higher than 60 mL/min per 1.73 $m^2$. However, an improvement is not observed in some categories of patients such as the elderly. Therefore, some authors challenged the supposed clinical improvement in the patient management brought by the CKD-EPI comparing with the MDRD[51]. In cirrhotic patients, studies that tested the CKD-EPI formula found a slightly better performance comparing with CG and MDRD although eGFR was higher than the mGFR in every study[34,48,49]. For example, in the study by Francoz *et al*[34] the mean bias was +8 mL/min per 1.73 $m^2$ *vs* +17 and +16 mL/min per 1.73 $m^2$ respectively for CG and MDRD4. However, the mean bias was similar to MDRD in patients with GFR below 70 mL/min per 1.73 $m^2$ (+19 mL/min per 1.73 $m^2$) suggesting strong overestimation of GFR in patients with CKD. Assessing the agreement by the mean of P30, our group recently found better results for CKD-EPI with the P30 being 56.4 % *vs* 42.6% for MDRD175[49]. However, a recent study highlighted the poor performance of the CKD-EPI formula in patients with severely decreased GFR (mean mGFR of 35.3 mL/min per 1.73 $m^2$) with low P30 at 6.8%, similar to those of MDRD[48]. Taken together, these data suggest that CKD-EPI may give a fairly good estimation of GFR in cirrhotic patients

with normal renal function. In patients with decreased renal function it presents the same limitations as CG and MDRD, which is mainly to overestimate the GFR.

## CYSTATIN C BASED FORMULA TO ESTIMATE GFR

These formulas were developed more recently. The mainly used formulas so far, are the Hoek[52] and the Larsson formula[53]. However, the Levey group published two new CKD-EPI formulas in 2012, one based on CysC (CKD-EPI CysC) and another based on both SCr and CysC (mixed CKD-EPI)[54]. While the CKD-EPI formulas were developed on a large group of patients, the Hoek formula was developed from a small group of 123 patients (Table 2).

In cirrhotic patients, some investigators tested the ability of these formulas to estimate the GFR appropriately[41,47,49]. Although one study, in a small group of patients with severely decreased renal function, found a poor performance of the Hoek formula (reflected by an important overestimation of the GFR and low accuracy with P30 of 11.4 %)[41], subsequent studies showed a better performance, at least comparing with SCr based formulas. In the study by Xirouchakis *et al*[47] P30 was observed to be 68%. Recent work by our group showed that the P30 could be even better at 78.7% but it dropped to 66.7% in patients with refractory ascites and to 53.8% in patients with GFR below 60 mL/min per 1.73 $m^2$. Nonetheless, performance remained higher than those of the SCr based formulas[49]. Concerning the two newly developed CKD-EPI formulas (CKD-EPI CysC and mixed CKD-EPI), we are the first to evaluate their performance in our recent study including 202 cirrhotic patient candidates for LT in whom an inulin renal clearance was performed[49]. We found that CKD-EPI CysC had the best performance compared to the other formulas tested in the study (*i.e.*, Hoek, MDRD 175, mixed CKD-EPI and "classic" CKD-EPI). The Hoek and CKD-EPI CysC formulas exhibited the lowest difference between eGFR and mGFR (respectively +4.3 and +4.4 mL/min per 1.73 $m^2$). However, the agreement, measured by the concordance correlation coefficient (CCC) and the P30, were improved for the CKD-EPI CysC formula (respectively 0.8 and 83.2% *vs* 0.75 and 78.7% for Hoek). Similarly to the Hoek formula, the P30 was lower in patients with refractory ascites (66.1%) and in case of GFR < 60 mL/min per 1.73 $m^2$ (73.1%). The ability to detect a GFR < 60 mL/min per 1.73 $m^2$ was the best for Hoek and CKD-EPI CysC formulas (both AUC of the ROC curve at 0.86). The ability to detect GFR < 90 mL/min per 1.73 $m^2$ was better in MDRD6 (0.77) and mixed CKD-EPI (0.78) formulas. Finally, regarding the new mixed CKD-EPI formula, its interest seems to be limited in cirrhotic patients with stage 3 to 5 CKD because of a poor agreement in cirrhotic patients with stage 3 to 5 CKD reflected by low P30 at 38.5% and overestimation of mGFR with a difference between mean eGFR and mean mGFR of +14.6 mL/min per 1.73 $m^2$. However, the mixed CKD-EPI performed similarly to CKD-EPI CysC in patients with refractory ascites (P30 = 63.9%) and even better than all other formulas in patients with GFR > 90 mL/min per 1.73 $m^2$ (P30 = 98.7% and P10 = 54.7%). In conclusion, these recent data suggest that the CysC based formula, especially the CKD-EPI CysC formula, yielded less biased eGFR than SCr based formulas, with a clear better performance in cirrhotic patients with CKD. Therefore, this formula should be used preferentially in cirrhotic patients with GFR < 60 mL/min per 1.73 $m^2$ and in those with refractory ascites. However, in patients with normal renal function, our results suggest that the mixed CKD-EPI has the best performance.

## CONCLUSION

Accurate and reliable assessment of GFR is warranted in cirrhotic patients in order to achieve optimal clinical management. Indeed, AKI and/or CKD are frequent complications in this context, impacting seriously on the prognosis of the patients. Moreover, several clinical conditions require the use of eGFR to adapt the treatment. Most of the available formulas estimating GFR exhibit limited suitability, particularly in case of a decreased renal function and/or severe cirrhosis, limiting their interest. The development of formulas based on CysC rather than SCr for estimating GFR opened the possibility to get a more robust and simple estimate of the GFR in daily clinical practice. Before developing a widespread use of CysC based eGFR in cirrhotic patients, however, further studies should be undertaken to confirm the clinical value of these formulas, especially those of the new CKD-EPI CysC.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

# Risk factors and outcome of bacterial infections in cirrhosis

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**Key words:** Immunity; Infection; Cirrhosis; Chronic liver disease; Bacterial translocation; Risk factors; Mortality

**Core tip:** We will discuss susceptibility and impact of specific bacterial infections in cirrhosis, their natural course and the identification of risk factors for organ failure and death in order to help clinicians identifying patients at the highest risk that may benefit from intensified surveillance, prophylaxis and therapy.

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## Abstract

Viable and non-viable pathological bacterial translocation promote a self-perpetuating circle of dysfunctional immune activation and systemic inflammation facilitating infections and organ failure in advanced cirrhosis. Bacterial infections and sepsis are now recognized as a distinct stage in the natural progression of chronic liver disease as they accelerate organ failure and contribute to the high mortality observed in decompensated cirrhosis. The increasing knowledge of structural, immunological and hemodynamic pathophysiology in advanced cirrhosis has not yet translated into significantly improved outcomes of bacterial infections over the last decades. Therefore, early identification of patients at the highest risk for developing infections and infection-related complications is required to tailor the currently available measures of surveillance, prophylaxis and therapy to the patients in need in order to improve the detrimental outcome of bacterial infections in cirrhosis.

## INTRODUCTION

Bacterial infections are diagnosed in 25% to 47% of hospitalized patients with cirrhosis[1-4] and represent the most important precipitating event for acute decompensation[5]. Infections are increasingly recognized as a major trigger of systemic inflammation and organ failure in advanced cirrhosis leading to a four-fold increased mortality[6]. Despite recent advances in understanding the underlying pathogenic mechanisms of bacterial infections in cirrhosis[7-9], the progression of infections to multiple organ failure and septic shock is still associated with a short-term mortality of patients exceeding 75%[5,10]. In this review we will discuss susceptibility and impact of specific bacterial infections in cirrhosis, their natural course and the identification of risk factors for organ failure and death in order to help clinicians identifying patients at the highest risk that may benefit from intensified surveillance, prophylaxis and therapy.

## PATHOLOGICAL BACTERIAL TRANSLOCATION

Decompensated liver cirrhosis predisposes to delayed intestinal transit time, increased intestinal permeability and disturbed expression of intestinal antimicrobial peptides thereby facilitating the translocation of bacteria and bacterial products from the gastro-intestinal lumen through the lamina propria into mesenteric lymph nodes (MLN), ascitic fluid and the systemic circulation[11-13]. Whereas bacterial translocation (BT) can be controlled by the immune system of healthy individuals, pathological BT in cirrhotic patients is accompanied by a cirrhosis-associated immune dysfunction (CAID) supporting systemic inflammation as a consequence of non-viable BT as well as bacterial infections due to viable BT[8,9,14].

Among the intestinal microbiota, Gram-negative enteric bacilli more easily translocate to MLN than Gram-positive bacteria and obligate anaerobes[15]. Although the capability to translocate across the gastrointestinal barrier varies between different *Escherichia coli* (*E. coli*) strains[15], no single virulence factor responsible for BT in cirrhosis could be identified yet[16]. Furthermore, catabolic and inflammatory stress to the epithelial barrier can increase BT of various coliform bacterial strains[17-19]. With classical bacterial culture techniques, enteric organisms can be detected in MLN from up to 30% of patients with Child-Pugh C cirrhosis compared to 9%-15% of non-cirrhotic patients undergoing laparotomy[20,21].

This increased translocation of intestinal bacteria and bacterial products in cirrhosis has been attributed to both clinically significant portal hypertension (PH) and advanced liver failure. The role of PH is supported by the observations that the development of ascites is a prerequisite for significant translocation of viable bacterial to the MLN in the CCl4 model[22], and that the use of non-selective beta blockers (NSBB) reduces the translocation of viable BT to MLN in cirrhotic rats[23] and prevents infectious complications in humans with decompensated cirrhosis[24]. Furthermore, it has been shown that non-viable BT and immune activation also occur in the pre-ascitic stage[25] and correlate with the increase in portal pressure[26]. On the other hand, pathological BT to MLN also occurs in models of experimental acute liver failure in the absence of PH[27] but not in chronic PH in the absence of cirrhosis[28]. Moreover, NSBB are also effective in preventing spontaneous bacterial peritonitis (SBP) in patients showing no hemodynamic response to treatment[24] underlining that PH may not be the principal driver of viable pathological BT in advanced cirrhosis.

Therefore, alternative mechanisms must be involved in triggering pathological BT in liver failure and advanced fibrosis. There is evidence that quantitative and qualitative changes in the intestinal microflora might contribute to this phenomenon. Small intestinal bacterial overgrowth (SIBO) frequently occurs in decompensated cirrhosis and correlates with systemic endotoxemia[29] and the presence of bacterial DNA fragments (bactDNA) in the peripheral blood[30]. Small intestinal dysmotility[31] and altered bile composition in cirrhosis[32] contribute to SIBO and drive endotoxemia and infectious complications[33]. Once a systemic or local immune response has been established, proinflammatory cytokines like tumor necrosis factor (TNF) and interleukin (IL)-6 disrupt the intestinal integrity of the intestinal barrier by altering the structure of the apical junctional complex[34,35] thereby further increasing intestinal permeability (IP) generally observed in cirrhosis[12,26]. Although some cohort studies show an association of IP with septic complications[12,36,37] the only prospective study available did not find an association of IP with infectious complications[38] questioning whether increased IP is really the cause - or rather the consequence - of pathological BT and intestinal inflammation in cirrhosis.

Whether the composition of the intestinal microbiome, modulates BT and infectious complications in cirrhosis has not been thoroughly investigated yet. There is some evidence that the fecal microbiome in cirrhosis is less diverse and shows an abundance of *Enterobacteriaceae*, *Streptococcus* spp. and *Enterococcus faecalis*[39-41], which mirrors the microbial pattern observed in SBP[1,42,43]. The microbiome associated with the colonic mucosa might be of particular interest because the enrichment of *Enterococcus, Proteus, Clostridium,* and *Burkholderia* and the loss of non-pathogenic commensal bacteria in cirrhosis was associated with more severe liver disease, increased inflammation and endothelial activation[44,45]. The recent findings that the gut microbiome is modulated by the enterocyte inflammasome[46,47] which leads to increased nonviable BT and augmented hepatic inflammation[48], suggest a critical interaction of gut mucosa, intestinal dysbiosis and immune activation in cirrhosis.

## IMMUNE DYSFUNCTION AND IMMUNE ACTIVATION IN CIRRHOSIS

To control viable BT in cirrhosis, immune control takes place at the epithelial barrier including the mucus layer, at gut-associated lymphatic tissue and the MLN and at the systemic level after bacteria or bacterial products have passed MLN *via* the portal vein or the thoracic duct[49]. In the context of pathological BT, organ-resident macrophages recognize pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), muramyl dipeptide, bacterial lipoproteins and bactDNA, *via* extra- and intracellular Toll-like receptors (TLR) (*e.g.*, TLR2, TLR4, TLR9) or intracellular NOD-like receptors (*e.g.*, NOD2). Ligation of these conserved pattern recognition receptors (PRR) leads to classical macrophage activation, the secretion of pro-inflammatory cytokines (TNF, IL-1β, IL-12) and polarization to pro-inflammatory macrophages supported by interferon (IFN)-γ released from activated T cells[50]. In healthy individuals, binding of LPS to TLR4 increases expression of scavenger receptors, MHC class Ⅱ and co-stimulatory molecules to acceler-

ate phagocytosis and subsequent presentation of bacteria and bacterial products[51]. Several abnormalities of the innate immune system in cirrhosis contribute to non-control of BT, subsequent inflammation and the increased incidence of infections. These abnormalities have been described as CAID syndrome (CAIDS) stressing the role of insufficient processing of bacteria and bacterial products by phagocytes[8]. Importantly, CAIDS is often not associated with inflammatory anergy but with marked immune activation and a high degree of systemic inflammation, which correlates with the severity of liver disease and predicts survival in these patients[52].

The liver itself plays a major role in phagocytizing pathogens and scavenging macromolecules because organ-resident Kupffer cells and liver sinusoidal endothelial cells account for 80% of the human reticuloendothelial system (RES)[53]. The overwhelming sinusoidal influx of bacterial components in liver cirrhosis owing to mechanisms described above, switches the physiological immune-modulatory state of the local hepatic immune system elicited by Kupffer cells to a state in which the production of pro-inflammatory cytokines predominates accumulating in further demise of hepatic parenchyma cells and deterioration of liver architecture[54]. Due to portosystemic shunting, decreased opsonization and dysfunctional phagocytic activity, the hepatic clearance function is markedly reduced in cirrhosis and correlates with the severity of liver disease, bacteremia and survival[55-58]. In parallel, non-classical pro-inflammatory subsets of monocytes expand and contribute to an inflammatory state in cirrhosis[52,59-61]. This state of dysfunctional activation has been observed in neutrophils as well. Neutrophils from patients with cirrhosis are potent producers of pro-inflammatory cytokines and reactive oxygen species but also display decreased chemotaxis and inefficient phagocytosis[62-67]. Dysfunctional phagocytosis and increased activation can be transmitted to neutrophils from healthy individuals with plasma from cirrhotic patients suggesting repetitive priming mediated by soluble factors[66,67], which may result in the cellular depletion of antioxidants and increased oxidative damage[65,68].

Whereas phenotypic and functional abnormalities have been well described for the aspects of the innate immunity in cirrhosis, the state of the adaptive immunity is less well defined. Phenotypically, patients with cirrhosis display decreased numbers of total and naïve T cells due to defective thymic generation and splenic sequestration in parallel with increased activation, proliferation and turnover of memory T cells presumably due to repetitive antigen stimulation[69,70]. Data on the functional consequences of these findings are sparse. Although clinical studies show attenuated immune responses after vaccination in cirrhotic patients[71-73], *in vitro* analyses could not identify specific T cell defects[74,75] but suggest soluble factors such as IL-10 as modulators of inconsistent T cell responses in cirrhosis[75-77].

Whether an observed depletion and functional alteration of innate natural killer (NK) cells[78,79] and memory B cells[79,80] in cirrhosis contribute to pathological BT, inflammation and bacterial infections in cirrhosis has not been demonstrated.

## ASSESSMENT OF SYSTEMIC INFLAMMATION IN CIRRHOSIS

In response to bacterial infection, patients with cirrhosis have a pronounced inflammatory response with elevated systemic concentrations of the pyrogenic cytokines IL-6 and TNF superimposing on high basal levels resulting in fever, leukocytosis and acute phase reaction[81]. As a consequence, systemic inflammatory response syndrome (SIRS) can be found in up to 67% of cirrhotic patients with bacterial infections compared to 37% of patients without, making sepsis a common complication of advanced cirrhosis[82-84].

Even in the absence of overt bacterial infection, the occurrence of systemic inflammation as indicated by SIRS is of prognostic relevance in patients with cirrhosis[83,85]. There is some evidence that beyond its role as an indicator of occult bacterial infection and advanced organ failure, systemic inflammation might even aggravate portal hypertension, renal failure and hepatic encephalopathy, thereby contributing to organ failure and death in cirrhotic patients by immunological, metabolic and hemodynamic mechanisms[83]. Due to hyperdynamic circulation with tachycardia or tense ascites leading to hyperventilation in patients with decompensated cirrhosis, SIRS criteria are most certainly less specific for inflammatory and infectious complications than in the general population and need to be interpreted with caution.

The magnitude of the acute phase response as indicated by C-reactive protein (CRP) or IL-6 levels might be more reliable tools than clinical SIRS criteria to estimate the risk for adverse outcome in cirrhotic patients. In patients with cirrhosis and SIRS concentrations of serum IL-6 correlate with organ failure, monocyte and neutrophil activation[86] but are also closely associated with the degree of portal hypertension[26] and hyperdynamic circulation[87]. The same observations have been made for CRP, where persistent elevation in the absence of infection indicates increased short-term mortality[88]. Discriminating infections from sterile systemic inflammatory response is only possible using CRP cut-offs are as high as 56 mg/L in advanced cirrhosis[89-91]. The large multicenter CANONIC study[5] identified elevated CRP levels and increasing WBC count as hallmark features to distinguish acute decompensation of cirrhosis from acute-on-chronic liver failure (ACLF) even when patients with bacterial infections were excluded. Patients who presented with ACLF had mean CRP levels of 33 mg/L (compared to 21 mg/L in patients without ACLF) and mean WBC counts of 9.4 Gpt/L (compared to 6.6 Gpt/L) at inclusion. Importantly, the probability of death in ACLF increased with the rise in WBC count[5] making WBC count an attractive, easily available linear variable to

assess mortality risk associated with systemic inflammation in cirrhosis.

Since none of these established indicators of inflammation allow a precise distinction between sterile SIRS and bacterial infection, treating physicians must be aware of risk factors of bacterial infections to assess the likelihood of bacterial infections in patients with advanced cirrhosis presenting with signs of systemic inflammation. We have recently evaluated novel biomarkers that more precisely indicate specific infections and/or immune activation in advanced cirrhosis, such as mid-regional pro-adrenomedullin and soluble urokinase plasminogen activator receptor[52,90], which will expand the diagnostic armamentarium for predicting outcome independent of the presence of confounding systemic inflammation.

## RISK FACTORS AND PROPHYLAXIS OF SPECIFIC BACTERIAL INFECTIONS IN CIRRHOSIS

SBP and spontaneous bacteremia are the most thoroughly investigated complications in patients with cirrhosis because they occur frequently with a prevalence of 15%-20% in hospitalized patients[65,92] and cause high mortality after one month (33%-42%) and after one year (49%-66%)[6]. Supporting the concept of pathological BT of Enterobacteriaceae in cirrhosis, Gram-negative bacteria have been isolated from more than 70% of culture-positive bacterial infections in the past[1,42]. More recently however, bacterial infections caused by Gram-positive cocci dramatically increased in tertiary centers and now represent 60% of nosocomial culture-positive infections including the archetypal infectious complications spontaneous bacteremia and SBP[3,37,43]. This shift has been attributed to the use of antibiotics leading to intestinal dysbiosis favoring Gram-positive BT, but also to increased invasive procedures and associated episodes of Gram-positive bacteremia with secondary organ spread[3,43,93].

As a preventive measure, primary antibiotic prophylaxis is currently recommended in cirrhotic patients with gastrointestinal (GI) bleeding because bleeding facilitates pathological BT and infections on the one hand, and infections are associated with a higher rate of recurrent bleeding on the other hand[94]. Antibiotic prophylaxis over seven days in cirrhotic patients in with gastrointestinal bleeding is the current standard of care[94,95]. In nine controlled trials including 987 patients the pooled incidence of bacteremia following GI bleeding was 15% without antibiotic prophylaxis and 3% with antibiotic prophylaxis, indicating a risk reduction of 75% with antibiotic prophylaxis[96]. Results favoring antibiotic prophylaxis were also obtained when the outcomes SBP, pneumonia, urinary tract infection, overall bacterial infections and mortality were analyzed separately[96,97]. Although translocation of gut microbial flora into the bloodstream is likely to occur during endoscopy because of mucosal trauma related to the endoscopy procedure itself[98], the risk of bacteremia associated with endoscopic procedures is poorly investigated in non-bleeding cirrhotic patients. One study reported an incidence of 10% (6/58) bacteremia by possible contaminants (Gram-positive skin flora) after colonoscopy, but all cirrhotic patients with bacteremia remained asymptomatic[99]. Thus, international guidelines do not recommend routine antibiotic prophylaxis for cirrhotic patients undergoing colonoscopy[100] because of the lack of data in this setting, and it is recommended that clinicians should decide "on an individual case basis[101].

### *Spontaneous bacterial peritonitis*

Patients with low ascitic fluid (AF) protein, elevated serum bilirubin levels and/or low platelets are at the highest risk of developing community-acquired SBP[102]. SBP has a recurrence rate of 70% within the first year after the first episode[103] making secondary prophylaxis with 400 mg norfloxacin daily a level A recommendation in current guidelines[95,104]. Because antibiotic prophylaxis in high-risk cirrhotic patients with low AF protein and with severe liver failure or with renal failure improves incidence of infections and short-term survival[105,106], primary antibiotic prophylaxis should be considered in these patients[95]. However, in the light of increasing antimicrobial resistance in cirrhotic patients and of decreased efficacy of antibiotic prophylaxis over time adherence to these guidelines is poor among practitioners resulting in cases of SBP that could have been prevented[107,108]. Genetic association studies may help identifying patients at the highest need for antibiotic prophylaxis. Frequent polymorphisms in genes involved in the innate antimicrobial defense, such as *NOD2*, *TLR2* and *MCP-1*, have been reported to confer a three- to four-fold increased life-time risk to develop SBP[109-113]. *NOD2* gene variants are of particular interest because they regulate intestinal immunity *via* expression of antimicrobial peptides and intracellular bacterial killing[114,115] thereby linking Paneth cell defense with pathological BT in cirrhosis[13,116]. In a recent study including four patients with *NOD2* variants, five patients with *TLR2* variants and 29 wild type controls, the presence of any of these variants was associated with markers of impaired intestinal permeability and higher systemic inflammation[26]. However, subsequent studies including a higher number of patients are required to answer the question on the influence of these gene variants on intestinal integrity and pathological BT as a driver of SBP *per se*. Whether genotype-based risk-stratification for antibiotic prophylaxis is a feasible approach to reduce infectious complications, at least in populations of European descent where these polymorphisms are frequently found, currently remains an open question but will hopefully be answered soon.

Whereas host factors contributing to SBP, such as genetic background or severity of liver disease[117], cannot be easily modified, environmental factors such as alcohol use in less advanced cirrhosis[118] or prescribed concurrent medication are more susceptible to therapeutic interven-

tion. In contrast to the protective effects of NSBB that have already been discussed above[24], the use of proton pump inhibitors (PPI) is associated with a three-fold increased risk for developing SBP in hospitalized patients according to a recent meta-analysis of observational studies[119] suggesting that the indication for PPI treatment in liver cirrhosis has to be very carefully weighed against the potential risks[120].

### Infections other than SBP

Studies on specific risk factors for extraperitoneal bacterial infections are scarce. Urinary tract infections (UTI) are very common and represent 20% up to 40% of bacterial infections in prospective studies of hospitalized patients with cirrhosis[2,121]. The majority of identified uropathogens are Gram-negative bacteria with *E. coli* as the most commonly isolated microorganism still, but multiresistant bacteria are increasingly observed, especially in nosocomial infection in southern Europe[4,84]. In a large retrospective cohort of almost 400 cirrhotic patients we could recently show that predominantly women develop UTI and that the risk of infection increases stronger with age than with the severity of underlying liver disease in contrast to other bacterial infections in cirrhosis[84]. Notably, in this and other series UTI was frequently accompanied by SIRS (42%-65% of cases)[83], and up to one third of patients with UTI presents with or develops concomitant bacterial infections[84,122] making UTI a bacterial infection that should not be underestimated in the cirrhotic patient. Although strategies for UTI prevention have not been investigated in the cirrhotic population specifically, evaluation and reduction of unnecessary urinary catheter use applies as in patients without liver disease.

Among the extraperitoneal manifestation of bacterial infections, lower respiratory tract infections and pneumonia are associated with the highest risk of mortality in cirrhosis[6,123]. Animal models suggest that low serum complement levels in rats with decompensated cirrhosis increase susceptibility to pulmonary infection with *Streptococcus pneumoniae* (*S. pneumoniae*)[124]. In agreement with these findings, patients with cirrhosis are more susceptible to pneumonia, present with a more complicated course of disease and develop more often bacteremia than non-cirrhotic patients[125,126]. Since *S. pneumoniae* represents the most common etiologic agent isolated in community-acquired pneumonia, pneumococcal vaccination with the 23-valent vaccine is recommended as a preventive measure despite reduced immunological responses and accelerated antibody decline in patients with cirrhosis[127,128].

Skin and soft tissue infections in cirrhosis can be caused by Gram-positive bacteria entering the edematous skin as well as by translocated Gram-negative bacteria. They are often recurrent, complicated by renal failure and lead to a mortality of approximately 20%[122,129,130]. Walking barefoot has been identified as a risk factor for cellulitis in patients with decompensated cirrhosis and edema by an Indian study and should be avoided[130].

Patients with cirrhosis have an increased risk of infectious endocarditis compared to the general population[131]. Infectious endocarditis in cirrhosis is predominantly hospital-acquired with *Staphylococcus aureus* (*S. aureus*), β-hemolytic streptococci and *Enterococcus* spp. as most frequent infectious agents, predominantly involves the aortic valve, and is associated with 60% renal failure and 50% mortality[132].

Bacterial Meningitis is a rare complication in cirrhosis (< 1%) often with atypical presentation and Gram-negative pathogen spectrum associated with bacteremia in the majority of cases and a mortality exceeding 40%[133,134].

## ESTIMATING THE OUTCOME OF BACTERIAL INFECTIONS

The meta-analysis by Arvaniti *et al*[6] comprising data from almost 12000 patients concluded a four-fold increased mortality owing to bacterial infections in cirrhotic patients - with respiratory tract infections, SBP and bacteremia as major contributors. We and others[84,123] could recently demonstrate that urinary tract infections in decompensated cirrhosis are less detrimental as these three major infections, but are still associated with a significantly increased risk of mortality, especially in the presence of concomitant SIRS. Fueled by excessive inflammation in cirrhosis, organ failure frequently occurs in the absence of septic shock, making bacterial infections a distinct stage in the natural history of cirrhosis progression[6] and the major precipitating event for the development of decompensation and ACLF[5]. There are three major factors that determine mortality of bacterial infection in cirrhosis irrespective of etiology: severity of underlying liver disease, concomitant renal failure and non-resolution of infection due to antimicrobial resistance.

### Severity of liver disease

Among the 18 variables that were significantly associated with death after bacterial infections in three or more studies evaluated by Arvaniti *et al*[6] five were related to liver function or severity of cirrhosis (Child-Pugh score, prothrombin time/INR, bilirubin, albumin, and MELD score) and three related to cirrhosis-associated complications (hepatic encephalopathy, gastrointestinal bleeding, and hepatocellular carcinoma). Therefore, using composite scores like Child-Pugh or MELD score to estimate prognosis is tempting, since they reflect severity of underlying disease as well as infection-triggered organ failure and are well-established scores to predict short-term and long-term mortality under a variety of conditions in cirrhosis. However, the independent contribution of systemic inflammation and bacterial infection to the deterioration of liver function needs to be considered: In the absence of SIRS a MELD score of > 18 is associated with 12% in-hospital mortality - in the presence of SIRS the mortality increases to 43%[83]; in the absence of leukocytosis a MELD score of ⩾ 22 is associated with

30% one-month mortality after SBP - in the presence of leukocytosis the mortality increases to 52%[135].

### Acute kidney injury and hepatorenal dysfunction

Clinically significant portal hypertension and pathological BT in cirrhosis contribute to mesenteric vasodilation and splanchnic pooling, which results in reduced central blood volume with compensatory but insufficient hyperdynamic circulation, activation of neurohumoral vasoconstrictor systems, and sodium retention in the kidneys[136,137]. The markedly reduced renal blood flow in decompensated cirrhosis[138] renders the kidney susceptible to infection-triggered renal failure and hepatorenal syndrome (HRS)[139]. Renal failure occurs in approximately one third of patients with cirrhosis and bacterial infections, especially after UTI, SBP, and skin infections, and is non-reversible or progressive in 25% to 33% of cases[121,140-142]. Compared to parenchymal nephropathy in cirrhosis, hypovolemia-related renal failure (OR = 2.32) and infection-related renal failure (OR = 2.61) are both associated with a two-fold increased risk of mortality within 90 d[143]. Given the large impact of progressive renal failure on mortality in patients with cirrhosis, current strategies implement the criteria of the Acute Kidney Injury Network (AKIN) [creatinine increase $\geqslant$ 0.3 mg/dL (27 μmol/L) within 48 h or 50% creatinine increase from a stable baseline] for all acute deteriorations of renal function in cirrhosis under the term "hepatorenal dysfunction"[144]. Patients with cirrhosis and infections that develop renal failure according to the AKIN criteria have lower mean arterial pressure, higher bilirubin, lower albumin, higher WBC count, higher platelet count, and lower serum sodium in univariate analyses[142]. Mortality increases with occurrence of AKI (34% *vs* 7% 90-d mortality), with severity of AKI (2%, 7%, and 21% in-hospital mortality in stages 1, 2, and 3, respectively) and with progression of renal failure (15% 90-d mortality after complete recovery, 40% after partial renal recovery, and 80% in patients without renal recovery or progression)[142,144].

Notably, one third of patients with AKI due to bacterial infections develop a second infection, which aggravates renal failure and reduces survival presumably by superimposing inflammation and exhaustion of innate defense mechanisms[65,122,142]. Therefore, prevention of secondary infection in cirrhotic patients with infection-induced renal failure seems a legitimate approach to reduce mortality due to infections. In addition, intravenous albumin substitution has proven effective to reduce renal failure, in-hospital mortality, and 90-d mortality in patients with SBP[145] and improves serum creatinine but not survival in cirrhotic patients with infections other than SBP[146]. Additional approaches using anti-inflammatory strategies with pentoxifylline have been proven effective in improving renal function during alcoholic hepatitis[147] and might have prophylactic potential for preventing HRS in decompensated cirrhosis in selected patients[148,149].

### Antimicrobial resistance

Failure of first-line empiric antibiotic therapy for bacterial infections in cirrhosis is associated with increased mortality, as it has been shown for SBP[43,150-152]. Since escalation of therapy after unsuccessful empiric therapy still carries an increased risk of mortality[151], knowing the patient's previous contact to the health-care system, used antibiotics, co-morbidities, and the local antibiotic susceptibility spectrum[153] is crucial for successful implementation of effective antimicrobial strategies. Whereas the frequency of multiresistant bacteria isolated from culture-positive bacterial infections in cirrhosis remained small for community-acquired infections, it has reached 40% in several tertiary hospitals around the globe resulting in a less than 50% resolution rate for nosocomial infections in cirrhosis[4,150,154]. Both, extended spectrum beta-lactamases (ESBL)-producing *Enterobacteriaceae* (predominantly in Southern Europe) and *Enterococcus* spp. (predominantly in Central Europe) are of increasing relevance in nosocomial SBP in Europe because both are associated with resistance to third-generation cephalosporins (TGC) which are currently used as the first line empiric therapy for community-acquired and healthcare-acquired SBP[43,150,151,154]. As a consequence, nosocomial acquisition has emerged as an important determinant for not surviving SBP, doubling the risk of mortality within 30 d[150].

Since colonization with ESBL-producing *Enterobacteriaceae* seems not to correlate with the development of TGC-resistant infections in cirrhosis[155], stool screening has currently no role for identifying hospitalized patients at risk. Therefore, clinicians are advised to carefully check the following risk factors for TGC-resistant and multiresistant bacteria in infected cirrhotic patients before initiating empiric antibiotic therapy: nosocomial acquisition, previous antibiotic treatment (norfloxacin prophylaxis, β-lactam use within the last three months), previous infection by multiresistant bacteria, diabetes mellitus, and upper GI bleeding[4,43,154]. Until now, no randomized controlled trials have evaluated the efficacy of empiric therapy with carbapenems, tigecycline, or addition of anti-enterococcal antibiotics (ampicillin, vancomycin) in the setting of hospital-acquired infections in cirrhosis. Therefore, clinicians are advised to implement the five main aspects summarized as the Tarragona strategy[153]: (1) Recognize individual risk factors; (2) Know local epidemiology; (3) Treat immediately and broad enough; (4) Select the ideal antibiotic for the site of infection; and (5) Re-evaluate your therapy after three days.

## CONCLUSION

Patients with decompensated cirrhosis represent a highly vulnerable population with structural, immunological and hemodynamic abnormalities, which render them susceptible to bacterial infections, pronounced systemic inflammation, organ failure, and death (Figure 1). Despite

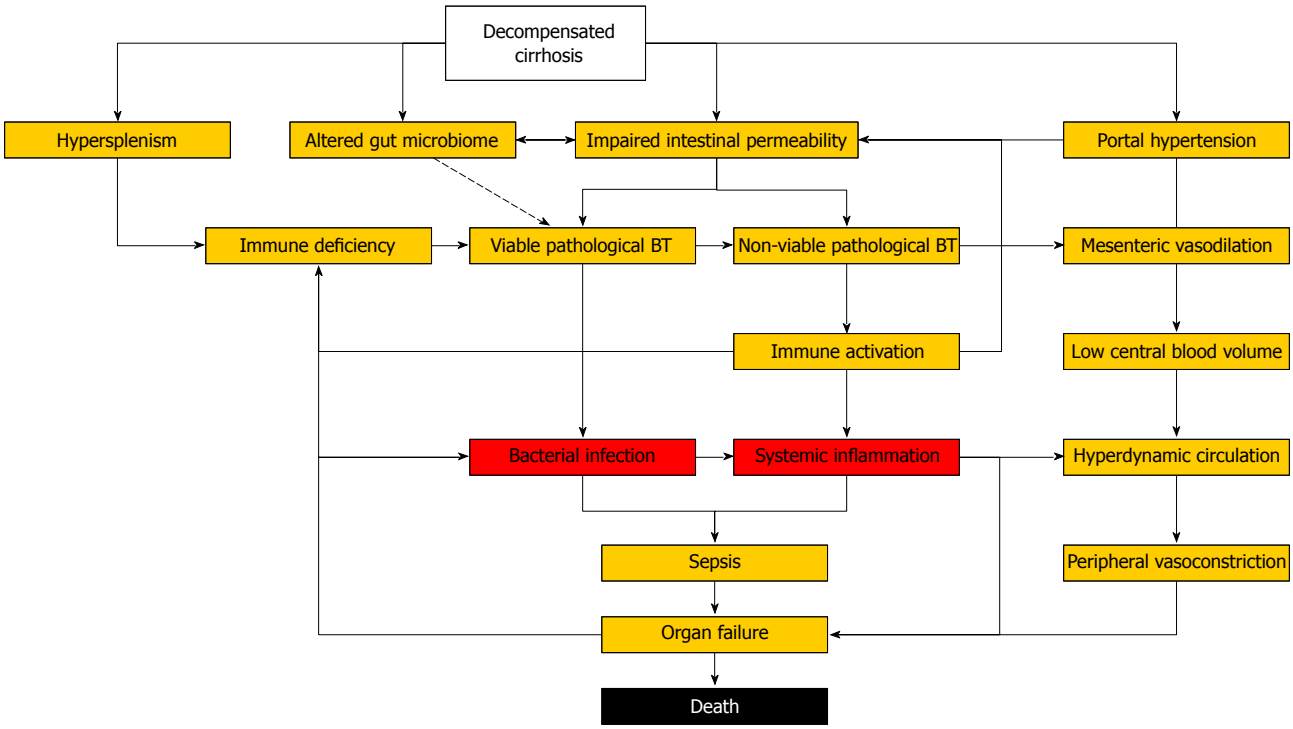


**Figure 1 Self-perpetuating mechanisms contributing to bacterial infections, systemic inflammation and organ failure in patients with decompensated cirrhosis.** BT: Bacterial translocation.

increasing understanding of underlying pathophysiology and mechanisms of organ failure, the outcome of bacterial infections in cirrhosis has remained poor over last decades. Antibacterial prophylaxis is effective in preventing infectious complications but increasing antimicrobial resistance demands its restriction to patients at the highest risk. Non-antibiotic approaches to decrease the risk of bacterial infections by reducing pathological BT (probiotics, prokinetics, bile acids) or restoring dysfunctional immune responses (anti-inflammatory strategies, immune therapy) are desperately needed as we enter the post-antibiotic era. Until then, basic measures of infection prevention (vaccinations, hygiene, nutrition), antimicrobial de-escalation strategies, and surveillance for early signs of organ dysfunction are required to reduce the burden associated with bacterial infections in cirrhosis.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

# Splanchnic vasodilation and hyperdynamic circulatory syndrome in cirrhosis

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## Abstract

Portal hypertension is a clinical syndrome which leads to several clinical complications, such as the formation and rupture of esophageal and/or gastric varices, ascites, hepatic encephalopathy and hepato-renal syndrome. In cirrhosis, the primary cause of the increase in portal pressure is the enhanced resistance to portal outflow. However, also an increase in splanchnic blood flow worsens and maintains portal hypertension. The vasodilatation of arterial splanchnic vessels and the opening of collateral circulation are the determinants of the increased splanchnic blood flow. Several vasoactive systems/substances, such as nitric oxide, cyclooxygenase-derivatives, carbon monoxide and endogenous cannabinoids are activated in portal hypertension and are responsible for the marked splanchnic vasodilatation. Moreover, an impaired reactivity to vasoconstrictor systems, such as the sympathetic nervous system, vasopressin, angiotensin Ⅱ and endothelin-1, plays a role in this process. The opening of collateral circulation occurs through the reperfusion and dilatation of preexisting vessels, but also through the generation of new vessels. Splanchnic vasodilatation leads to the onset of the hyperdynamic circulatory syndrome, a syndrome which occurs in patients with portal hypertension and is characterized by increased cardiac output and heart rate, and decreased systemic vascular resistance with low arterial blood pressure. Understanding the pathophysiology of splanchnic vasodilatation and hyperdynamic circulatory syndrome is mandatory for the prevention and treatment of portal hypertension and its severe complications.



**Key words:** Portal hypertension; Splanchnic flow; Splenic circulation; Nitric oxide; Autonomic dysfunction; Cirrhosis; Hyperdynamic circulatory syndrome

**Core tip:** In cirrhosis, portal hypertension is due to an increase in intrahepatic resistance and splanchnic blood flow. The latter is secondary to arterial splanchnic vasodilatation and the opening of collateral circulation. Though the increase in intrahepatic resistance is the earliest and most important component, at present the only treatment regimes which are available for the control of portal hypertension in cirrhosis, *i.e.*, non-selective beta-blockers, octreotide and terlipressin, act on the splanchnic dynamic component. Thus, understanding the mechanisms that lead to splanchnic vasodilatation and to the hyperdynamic circulatory syndrome is essential for the treatment of the complications of portal hypertension.

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## INTRODUCTION

Portal hypertension is a clinical syndrome responsible for

the onset of serious clinical complications such as the formation and rupture of esophageal and/or gastric varices, ascites, and hepatic encephalopathy.

In cirrhosis, the main mechanism responsible for the increase in portal pressure is the increase in intrahepatic resistance to portal blood outflow.

The pivotal mechanism responsible for the increased resistance in cirrhosis is the deposition of collagen in the hepatic acinus with narrowing of the sinusoidal lumen and a consequent decrease in the total cross-sectional area of the hepatic sinusoids. A further structural change responsible for the increase in intrahepatic portal resistance is the compression of centrilobular venules by regenerating nodules, granulomas, and portal inflammation. The main role of such anatomical alterations in determining the increase in portal pressure is confirmed by the relationships between septal thickness, small nodularity, liver stiffness and portal pressure[1,2].

Apart from the structural component, a vasoactive, potentially reversible component is also involved in the increase in hepatic resistance[3]. In cirrhosis, the contractile tone of smooth muscle cells and myofibroblasts, derived from stellate cells, around the sinusoids and hepatic venules is increased[4]. Norepinephrine, substance P, thrombin, angiotensin Ⅱ[5], endothelin (ET)[6] and prostanoids[7] increase the contractile tone of myofibroblasts and thus portal resistance. Nevertheless, endothelial dysfunction is the main source of the dynamic increase in intrahepatic portal resistance[8]. A decreased bioavailability of nitric oxide (NO) in the sinusoids[8-11] and an increased production of cyclooxygenase (COX)-derived prostanoids, such as prostaglandin H2 and tromboxane A2[7,12] seem to be the main players in endothelial dysfunction in cirrhosis.

Despite being crucial to the development of hemodynamic changes in cirrhosis, the mechanisms responsible for the increase in intra-hepatic resistance will not be analyzed in details in this review, as they are beyond its aim.

## INCREASED SPLANCHNIC INFLOW

Due to increased intra-hepatic resistance, a reduction in portal blood flow volume may be expected in portal hypertension. However, while a dilatation of the portal vein and a decrease in portal blood velocity are detected[13], these patients are characterized by a net increase in portal, splenic and mesenteric inflow.

The opening of portal-systemic collateral circulation participates in the increase in portal inflow. However a primary splanchnic arterial vasodilation can also be observed, with increased splenic and mesenteric blood flow.

Portal pressure results from the relationship between the blood flow volume entering the portal system and the resistance to outflow of portal blood. The mathematical expression of this relationship is provided by Ohm's formula: P = Q * S, where P represents change in pressure along the vessel, Q represents blood flow and R resistance to the flow.

The increase in resistance to portal flow is the main determinant of portal hypertension in cirrhosis[14,15], but an increase in portal inflow also plays a role. Such increase in total splanchnic inflow[16,17] has been observed in patients with cirrhosis and demonstrated in experimental models of portal hypertension[18,19].

In the liver with normal resistance, a change in portal flow does not change portal pressure[20] because of the high vascular compliance of hepatic vasculature. When outflow portal resistance is increased and vascular compliance is decreased, an increase in portal flow is responsible for an increase in portal pressure. In cirrhosis, the increase in portal inflow, which is triggered by the increase in resistance to portal flow (see below), maintains and worsens portal hypertension[21].

Several therapeutic strategies for portal hypertension aim at decreasing portal pressure by decreasing portal inflow, thus highlighting the pathogenic role of portal inflow.

## PORTAL-SYSTEMIC COLLATERALS

A mechanism explaining the maintenance of a high portal inflow in portal hypertension is the opening of portal-systemic collaterals, caused by the increase in resistance to outflow from the portal system.

The opening of collateral circulation occurs through the reperfusion and dilatation of preexisting vessels, but also through the generation of new vessels, as it was demonstrated by experimental studies showing the role of angiogenetic factors such as vascular endothelial growth factor (VEGF) in the pathogenesis of collateral circulation in portal hypertensive rats[22,23].

Portal-systemic shunts are responsible for gastrointestinal hemorrhage (mostly due to the rupture of esophageal or gastric varices) and allow access to the systemic circulation of substances which are usually removed by the liver. These play a role in the pathogenesis of the hyperdynamic circulation, ascites and hepatic encephalopathy[24].

## SPLANCHNIC VASODILATION

In cirrhosis with portal hypertension, the increase in splanchnic blood flow is caused by a vasodilation of arterial splanchnic vessels, both in splenic and mesenteric vascular beds. In recent years, the mechanisms responsible for the reduction in mesenteric arterial resistance in cirrhosis have been extensively investigated. Numerous substances and systems have been proposed as possible mediators: glucagon[24-26], prostacyclin (PGI2), intestinal vasoactive peptide, histamine, substance P, estrogens, colecystokinin, ammonia, endotoxins, adenosine, biliary acids[24], NO[27-29], alpha-calcitonin gene-related peptide[30], adrenomedullin[31,32], VEGF[33], carbon monoxide (CO)[34,35], endogenous cannabinoids (ECs)[24,28]. Endothelial factors certainly play a major role[36].

The role of NO in the splanchnic vasodilatation of

patients with cirrhosis and portal hypertension has been hypothesized more than 20 years ago[37] and nowadays it is considered the pivotal factor involved in the decreased mesenteric resistance of cirrhosis. NO has a very short half-life (20-30 s), it diffuses freely through the cellular membrane and acts mainly by increasing the production of cGMP by guanylate cyclase with subsequent relaxation of the smooth muscle cells. NO bioavailability is increased in patients with cirrhosis and portal hypertension[38], mostly because of an increased activity of the constitutive form of NO synthase (eNOS).

NOS activity is increased in the superior mesenteric artery and thoracic aorta of portal hypertensive rats[39]. Upregulation of eNOS can be detected even in early phases of the disease in portal hypertensive rats[35]. Several mechanisms seem to be responsible for such increase in eNOS activity. Inflammatory cytokines, VEGF and mechanical forces such as shear stress induce signaling cascades to activate Akt and heat shock protein 90 (Hsp90) which, in turn, activate eNOS[40]. Bacterial translocation from the gut into mesenteric lymph nodes is another early mechanism increasing tumor necrosis factor-alpha, eNOS cofactor tetrahydrobiopterin and eNOS-derived NO[41].

In decompensated cirrhosis, also the inducible form of NOS (iNOS) is upregulated in mesenteric arteries[42,43], even though its active role in the increased bioavailability of NO in the mesenteric vascular bed has not been clearly demonstrated. NO is also an angiogenic factor and it may play a role in the increased splanchnic angiogenesis which characterizes portal hypertensive rats[44]. In experimental cirrhosis with portal hypertension increased mesenteric angiogenesis can be reversed by chronic inhibition of NO formation[45].

However, NO/eNOS is not the only system involved in the mesenteric vasodilation of cirrhosis. Indeed, chronic administration of a NOS inhibitor in ascitic cirrhotic rats only partially corrects the mesenteric vasodilatation[35,43], thus implicating other vasoactive systems in the decrease in mesenteric resistance. PGI2, an endogenous vasodilator produced by vascular endothelial cells, is increased in patients with cirrhosis[46]. In portal hypertensive rats, an enhanced COX-1 expression within the superior mesenteric artery has been shown[47]. The inhibition of COX by indomethacin reduces portal pressure, improves hyperdynamic circulation[48,49] and reduces splanchnic blood flow[47].

It has been observed that in the superior mesenteric artery of cirrhotic rats treated with both NOS and COX inhibitors, an endothelium derived hyperpolarizing factor (EDHF) can replace NO and PGI2, inducing arterial dilation[50]. The exact nature of EDHF is controversial; however, arachidonic acid metabolites, the cation $K^+$ and hydrogen peroxide are the main potential candidates[51]. A study conducted by our group suggested that in cirrhosis the increased NO/PGI2-indipendent vasodilation of mesenteric arteries is due, at least in part, to excess reactivity to 11,12-epoxyeicosatrienoic acid through an increased expression of myoendothelial gap junctions[52].

Also CO, an end product of heme catabolism by heme oxygenase (HO), is involved in the splanchnic vasodilatation in cirrhosis. CO, like NO, is an activator of guanyl cyclase and of large-conductance calcium-activated potassium channels (BKCa). It has been hypothesized that NO and CO may act in a coordinated fashion to maintain the patency of the sinusoids as a reaction to the upregulation of sinusoidal constrictors such as ET[53]. In a cirrhotic liver, the downregulation of NOS activity may not be compensated by a sufficient upregulation of other dilators, such as CO, with an increase in sinusoidal resistance as a result. On the other hand, in the aorta[54] and in the mesenteric arteries[35,54-56] of cirrhotic rats, the expression of HO-1 is increased. Moreover, the inhibition of HO improves the hyperdynamic circulatory syndrome[54] and normalizes the response of mesenteric arteries to phenylephrine in cirrhotic rats with ascites[35]. These data suggest that in cirrhosis the HO/CO system may play a role in the splanchnic hemodynamic alterations, especially in the later stages of the disease. In mesenteric arteries of ascitic cirrhotic rats, the increased levels of CO due to HO upregulation induce the expression of BKCa[56]. In cirrhotic rats with ascites, the upregulation of BKCa may explain the hypersensitivity of mesenteric arteries to CO[56].

ECs are ubiquitous lipid signaling molecules that determine central and peripheral effects through specific receptors CB1 and CB2. Experimental data suggest that ECs contribute to the development of splanchnic vasodilatation and portal hypertension by overactivating CB1 receptors within the mesenteric vasculature[51,57-59]. The effects of ECs on the splanchnic vessels seems to be due both to an increase in NO production[57] and to a NO-independent mechanism, since in isolated mesenteric arteries from cirrhotic rats endothelial denudation did not abolish the vasodilating effect[60].

In patients with chronic liver disease, autonomic dysfunction has also been observed and peripheral vasodilatation is probably also a consequence of a decreased reactivity to vasoconstrictor systems, such as the sympathetic nervous system[61], vasopressin, angiotensin Ⅱ and ET-1. Autonomic dysfunction has been often attributed to alcohol-related neuropathy, but other mechanisms may also be involved as neuropathy is more common in in chronic alcohol misusers with liver damage than in their conterparts without liver damage[62]. A downregulation or a decreased affinity of the receptors for vasoconstrictors may explain the sustained systemic and splanchnic vasodilatation that occurs despite the activation of the vasoconstrictor systems. Finally, a downregulation or an impaired vasoconstrictor-induced activation of Rho kinase may represent a further mechanism contributing to defective contractility in cirrhosis[63].

## SPLENIC CIRCULATION AND PORTAL HYPERTENSION

In cirrhosis with portal hypertension, the increase in mes-

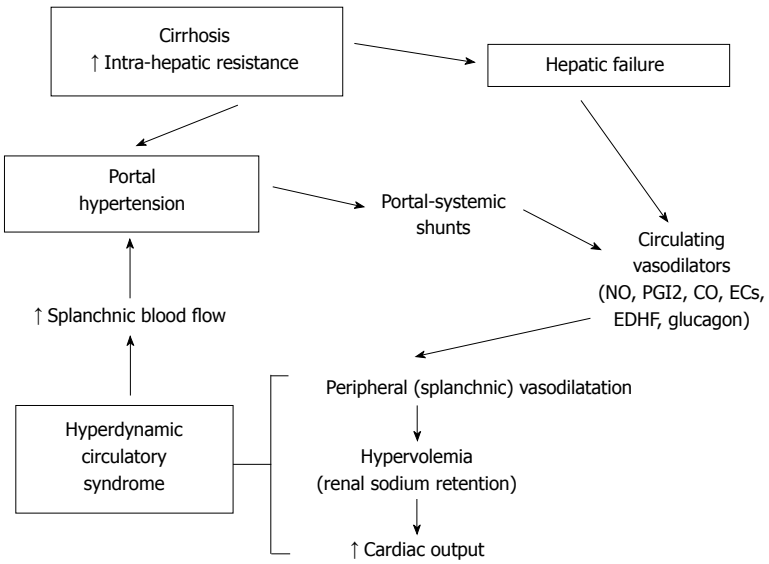


**Figure 1 Schema of the relationships among splanchnic vasodilation, hyperdynamic circulatory syndrome and portal hypertension in liver cirrhosis.** NO: Nitric oxide; PGI2: Prostacyclin; CO: Carbon monoxide; ECs: Endogenous cannabinoids; EDHF: Endothelium derived hyperpolarizing factor.

enteric blood flow is not the only factor determining an increase in portal blood inflow, since splenic blood flow is also enhanced[64]. Indeed, it has been observed that in patients with cirrhosis and portal hypertension splenic blood flow increases to a higher extent than mesenteric flow[65,66]. While in healthy subjects the spleen contributes to portal blood flow for only about 40%[24], in patients with cirrhosis the splenic component represents more than 50% of portal inflow[67]. This increase in splenic blood flow has been observed in patients with cirrhosis and splenomegaly[64,68]. Moreover, a correlation exists between spleen size and portal vein diameter[69,70], splenic blood flow[71,72] and portal blood flow[69,73].

These data suggest that in cirrhosis the splenic congestion is not a passive phenomenon and the spleen actively contributes in maintaining portal hypertension by congesting the portal system[17].

## HYPERDYNAMIC CIRCULATORY SYNDROME

In cirrhosis, the hyperdynamic circulation is characterized by increased cardiac output and heart rate, and decreased systemic vascular resistance with low arterial blood pressure[74,75]. The main cause of the onset of the syndrome is the systemic and splanchnic vasodilatation, which eventually leads to abnormalities in the cardiovascular system, and several regional vascular beds. Chronic administration of a NOS inhibitor in ascitic cirrhotic rats completely normalized the parameters of hyperdynamic circulation[76], but only partially corrected the mesenteric vasodilatation[35,43]. Neuropeptide Y, a sympathetic co-transmitter of norepinephrine, exerted marked portal hypotensive effects and ameliorated the hyperdynamic circulation through a pronounced splanchnic vasoconstriction and a reduction in splanchnic blood flow in cirrhotic rats with ascites[77].

Collateral circulation contributes to the development of hyperdynamic syndrome, both directly, by reducing peripheral resistance, and indirectly, by allowing intestinal vasoactive substances to bypass the liver and reach the systemic circulation. Neurogenic, biochemical and local mechanisms are also involved in the progression of the syndrome[21]. Even if overall peripheral and splanchnic vascular resistance is markedly reduced, a decrease in resistance is not present in all vascular beds[78]. A blood flow reduction has been observed in the kidney[79-82], in the brain[83-86] and in muscles[87]. The more the liver disease and the splanchnic vasodilatation worsen, the more the blood flow to other organs decreases[78]. Renal vasoconstriction is a consequence of effective hypovolemia and the activation of neurohumoral systems, providing the rationale for improving renal blood flow not by renal vasodilators, but by albumin infusion and splanchnic vasoconstrictors such as terlipressin or octreotide[88-91].

The hyperdynamic circulatory syndrome is the pathogenetic basis for the development of several complications of cirrhosis, such as hepato-renal syndrome, hepato-pulmonary syndrome, shock susceptibility and tissue hypoxemia. Moreover, since the gut and liver receive a third of the entire cardiac output, hyperdynamic circulation directly or indirectly contributes to two of the most serious complications of cirrhosis: ascites and variceal bleeding. Also, several symptoms of portal hypertension (*i.e.*, tachycardia, subcyanotic warm skin, systemic arterial hypotension) are a direct consequence of this syndrome. Another main finding in the hyperdynamic circulatory syndrome is the increase in circulating blood volume[92-95]. According to the "peripheral arterial vasodilatation theory"[96], splanchnic vasodilatation leads to renal sodium retention and, as a consequence, hypervolemia which contributes to the hyperdynamic syndrome by increasing cardiac pre-load. The hyperdynamic syndrome, in turn, maintains and enhances portal hypertension, while anti-aldosteronic drugs reduce portal pressure in cirrhotic patients without ascites by decreasing blood volume[97,98] (Figure 1). The hyperdynamic circulatory syndrome only develops in the supine position, in which a portion of

blood volume is moved towards the "central" area[99,100]. In cirrhosis, the increase in cardiac output with compensatory reduction in peripheral vascular resistance is a consequence of the enhanced cardiac pre-load due to the supine posture[99,100].

In patients with cirrhosis, a higher cardiac output is independently associated with higher hepatic venous pressure gradient and hepatic blood flow[101]. Despite the increase in the baseline cardiac output, the systolic and diastolic ventricular response to stress is blunted in conjunction with ventricular hypertrophy or dilatation and electrophysiological abnormalities such as prolonged QT interval. The following mechanisms seem to be involved in the development of cirrhotic cardiomyopathy: an altered β-adrenergic signalling[102,103], a dysfunction of the cardiomyocytes membrane, and an activation of cardiodepressant substances such as NO, cytokines and ECs[104].

## PHARMACOLOGICAL APPROACHES TO SPLANCHNIC VASODILATATION AND HYPERDYNAMIC CIRCULATORY SYNDROME

Several therapeutic strategies for portal hypertension complications aim at decreasing portal pressure by decreasing portal inflow, thus highlighting the pathogenic role of splanchnic vasodilatation and hyperdynamic circulatory syndrome.

In the latest Baveno conference (May 2010), the therapeutic recommendations for non-selective β-blockers included primary prophylaxis of bleeding from gastroesophageal varices and the prevention of recurrent hemorrhage[105]. The efficacy of non selective beta-blockers in patients with portal hypertension is due to the fact that they increase splanchnic resistance and decrease cardiac output, thus decreasing portal inflow[106].

Vasopressin analogues and somatostatin are the most widely utilized vasoactive drugs during an acute variceal bleeding. Terlipressin, a synthetic vasopressin analogue, causes splanchnic and systemic vasoconstriction by acting on the V1a receptors within the arterial smooth muscle. Somatostatin and its analogues cause splanchnic vasoconstriction both by inhibiting the release of the vasodilator glucagon, and through a direct mesenteric vasoconstrictive effect. Vasopressin analogues and somatostatin also blunt the increase in portal pressure induced by volume expansion[107,108]. Splanchnic vasodilation is responsible for the hypoperfusion of the renal system which leads to the activation of the renin-angiotensin-aldosterone system and to fluid retention. Diuretics are the pivotal drugs in the control of ascites. The standard combination includes spironolactone, an aldosterone antagonist, and furosemide[109]. In patients with ascites, albumin is used to prevent paracentesis-induced circulatory dysfunction, or an acute worsening of the hyperdynamic circulatory syndrome with a marked increase in plasma renin activity and plasma norepinephrine that occurs after a large volume paracentesis[110]. Also terlipressin and midodrine, an alpha-1 agonist upon arterial and venous vessels, seem to be effective in reducing the manifestations of paracentesis-related circulatory dysfunction[111,112]. Moreover, in refractory or recurrent ascites, midodrine significantly decreased cardiac output and increased systemic vascular resistance, thus being superior to standard medical therapy alone in controlling ascites[113]. In patients with hepatorenal syndrome, the rationale for the use of terlipressin, norepinephrine or midodrine in association with albumin is to counteract the splanchnic arterial vasodilation, thus increasing the effective circulating volume and, in turn, renal perfusion[114,115].

## CONCLUSION

In cirrhosis, the increase in portal pressure which is responsible for the onset of complications such as gastrointestinal hemorrhage, ascites, hepatic encephalopathy, hepato-renal syndrome, hepato-pulmonary syndrome and spontaneous bacterial peritonitis, is due not only to an enhanced intrahepatic resistance to portal blood outflow, but also to an increase in splanchnic blood inflow into the portal vascular system.

The increase in splanchnic blood flow is determined by a vasodilatation of the arterial splanchnic vessels, both in the splenic and mesenteric vascular beds, and by the development of collateral circulation. The increase in NO production in the splanchnic vascular bed is considered the main contributor to splanchnic vasodilatation. However, several others molecules, such as PGI2, EDHF, CO and ECs also play a role. Splanchnic vasodilatation leads to the development of hyperdynamic circulatory syndrome, which is characterized by increased cardiac output and heart rate, and decreased systemic vascular resistance with low arterial blood pressure. The understanding of the pathophysiology of splanchnic vasodilatation and hyperdynamic circulatory syndrome is essential for the prevention and treatment of the complications of portal hypertension.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

# Immune dysfunction in cirrhosis

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## Abstract

Innate and adaptive immune dysfunction, also referred to as cirrhosis-associated immune dysfunction syndrome, is a major component of cirrhosis, and plays a pivotal role in the pathogenesis of both the acute and chronic worsening of liver function. During the evolution of the disease, acute decompensation events associated with organ failure(s), so-called acute-on chronic liver failure, and chronic decompensation with progression of liver fibrosis and also development of disease specific complications, comprise distinct clinical entities with different immunopathology mechanisms. Enhanced bacterial translocation associated with systemic endotoxemia and increased occurrence of systemic bacterial infections have substantial impacts on both clinical situations. Acute and chronic exposure to bacteria and/or their products, however, can result in variable clinical consequences. The immune status of patients is not constant during the illness; consequently, alterations of the balance between pro- and anti-inflammatory processes result in very different dynamic courses. In this review we give a detailed overview of acquired immune dysfunction and its consequences for cirrhosis. We demonstrate the substantial influence of inherited innate immune dysfunction on acute and chronic inflammatory processes in cirrhosis caused by the pre-existing acquired immune dysfunction with limited compensatory mechanisms. Moreover, we highlight the current facts and future perspectives of how the assessment of immune dysfunction can assist clinicians in everyday practical decision-making when establishing treatment and care strategies for the patients with end-stage liver disease. Early and efficient recognition of inappropriate performance of the immune system is essential for overcoming complications, delaying progression and reducing mortality.



**Key words:** Cirrhosis; Immune dysfunction; Endotoxemia

**Core tip:** Innate and adaptive immune dysfunction, also referred to as cirrhosis-associated immune dysfunction syndrome, plays a pivotal role in the pathogenesis of cirrhosis in both acute and chronic disease progression. During progression, acute decompensation is associated with organ failure(s), the so-called acute-on chronic liver failure, and chronic decompensation with progression of liver fibrosis and development of disease specific complications comprise distinct clinical entities with different immunopathology mechanisms. Enhanced bacterial translocation associated with systemic endotoxemia and systemic bacterial infections have substantial impacts in both clinical situations. In this review the authors provide overview of immune dysfunction and its consequences in cirrhosis.

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## INTRODUCTION

Cirrhosis is the final stage of chronic liver diseases from any cause and is associated with various levels of immune dysfunction, which are referred to as cirrhosis-associated immune dysfunction syndrome (CAIDS)[1]. Acquired alterations of both the innate and the adaptive immune functions are diverse, encompassing recognition, effector and regulatory mechanisms[2]. Paradoxically, depression and overstimulation exist concurrently in the system, and result in an enhanced susceptibility to acute inflammatory processes and their exaggerated courses, both locally and far from the portal of entry of the microbes or the non-microbial toxic agents. The worst consequence of the imbalance in the pro- and anti-inflammatory processes is the development of acute-on-chronic liver failure (ACLF). Subtle immune dysfunction, however, also favors a shift towards persistence of inflammation leading to progression of liver fibrosis and development of different complications (portal hypertension and hepatic encephalopathy). From a pathogenetic point of view, the predominant mechanisms are different during acute and chronic worsening of liver function in cirrhosis[3]. Enhanced bacterial translocation (BT)[4] associated with systemic endotoxemia and increased occurrence of systemic bacterial infections have substantial impacts on both clinical situations[5]. The other important feature is that the immune status of patients is not constant during the illness, and the extent of the acquired immune dysfunction is related to the severity and etiology of the liver disease. The more severe the liver disease, the more subtle is the immune dysfunction[6]. In the case of an alcoholic etiology, more profound alterations are generally expected[7]. Lastly, in cirrhosis, the clinical effect of functional variations of innate immunity-related genes are more pronounced compared to non-cirrhotic cases because of a pre-existing acquired immune dysfunction with limited compensatory mechanisms.

## INNATE IMMUNE DYSFUNCTION

### *Pattern recognition receptors*

Different classes of germ line-encoded pattern recognition receptors (PRRs) recognize invading pathogens, and monitor the extracellular and intracellular compartments of host cells for signs of microbes. Sequential detection of a pathogen by various PRRs in different subcellular compartments is essential and results in activation and the complex interplay of downstream, conserved signaling pathways[8]. PRRs are widely distributed in different forms with various functions all over the human body. They are abundant at the sites of possible entry for pathogenic microorganisms. PRRs are anchored in innate immune cells as surface or intracellular receptors, and are involved in signaling, resulting in an inflammatory response and subsequent cellular activation. The other type of PRRs includes various soluble receptors that move around freely and are considered as functional ancestors of the immunoglobulins (Ig). They act as phagocytic receptors, mediating direct non-opsonic uptake of pathogenic microbes and/or their products. On the basis of their function, scavenger receptors (SR), which are cell membrane-bound PRRs, also belong to this latter group[8]. These molecules recognize conserved structures, designated pathogen-associated molecular patterns (PAMPs), on microbes. Many of these molecules are present in commensals and opportunistic pathogens (MAMPs, microbial-associated molecular patterns)[9]. Moreover, PRRs interact not only with exogenous microbial molecules, but also with endogenous structures. Damaged or stressed cells that pose a "danger" to self-tissues are recognized through danger (or damage)-associated molecular patterns (DAMPs)[10]. A multifaceted interplay of different PRRs results in a complex spectrum of pro- and anti-inflammatory, immunogenic and suppressive responses induced within the host.

Altered expression and function of the PRRs are well-known features of cirrhosis. Of the acquired alterations in toll-like receptors (TLRs), PRRs are the most extensively studied and are reported to have a substantial impact on the pathogenesis and evolution of the disease[11,12]. Recently, interesting data has been revealed about other PRRs, such as the cluster of differentiation 14 (CD14)[13], macrophage SR, soluble(s) CD163[14], or C-type lectin receptors[15].

### *Altered TLR expression and functions*

A wide range of TLRs is expressed to various extents in the liver parenchymal and non-parenchymal cells[12,16,17]. Acquired alterations in TLR signaling pathway have a major influence on the development of the disease and have been extensively studied in cirrhosis[18]. Previous experimental studies on animals, mostly rodent models of liver fibrosis mimicking different etiologies of chronic liver diseases (CLD)[17,19] and models with knock-outs of certain members of cell signaling molecules, delineated the most relevant signaling routes involved in the pathogenesis of fibrosis, namely the TLR2, 4 and 9 pathways[20]. TLR2 and TLR9 recognize their ligands, di- and triacyl lipoproteins and unmethylated CpG-DNA, respectively, while the lipid A component of lipopolysaccharide (LPS) triggers the activation of TLR4[11,12,21]. The above mentioned animal models also highlighted that hepatic stellate cells (HSCs) are the ultimate effectors of TLR ligand-mediated fibrogenesis in the liver[22] and that maintenance of liver homeostasis depends upon the summation of pro- and anti-fibrotic effects of various immune cells on HSCs. Profibrotic immune cells, like M1 macrophages, neutrophils, T helper (Th) 17 cells, $CD8^+$ T cells and nature killer T (NKT) cells, promote fibrosis *via* secretion

of proinflammatory cytokines and mediators activating HSCs, while secretion of interleukin (IL)-10 and IL-22, interferon gamma (IFNγ), tumor necrosis factor related apoptosis inducing ligand (TRAIL), and direct killing of HSCs by anti-fibrotic immune cells (M2 macrophages, $CD11b^+Gr1^+$ bone marrow cells, regulatory T cells (Treg), Th17 cells, NK cells and NKT cells) can negatively regulate HSCs. Interestingly, macrophages, NKT cells, Th17 cells and dendritic cells seem to possess dual functions in this regard[23]. Thus, NK cell-mediated elimination of activated HSCs is a key component of maintaining liver homeostasis and preventing fibrogenesis, principally in the early stages of liver fibrosis[24,25].

Changes in TLR signaling pathways are caused by the prolonged exposure to intestine-derived bacterial products (LPS, unmethylated CpG containing DNA and lipoteichoic acid), foreign toxic agents (ethanol and acetaldehyde derived adducts) and also damaged hepatocyte-derived endogenous TLR ligands[26], which are well-established components of CAIDS[1]. Intestinal bacterial overgrowth, altered composition of the gut microbiome, bowel dysmotility, impaired local intestinal mucosal immunity and multifactorial disruption of the intestinal mucosa barrier (increased oxidative stress, mucosal edema and consequential mucosal structural changes causing an enhanced intestinal permeability) ,together result in pathological BT in cirrhosis[4,27]. Moreover, the decreased capacity of the liver to filter these bacterial products by hepatic resident macrophages [Kuppfer cells (KC)] and reduced LPS scavenging capacity of albumin caused by oxidization[28] and low levels of high density lipoprotein (HDL) and apolipoprotein A- I [29], further assist the elevation of the above-mentioned, potentially immunogenic bacterial products in the systemic circulation. Attenuation or complete inhibition of LPS/TLR4 pathways by either intestinal decontamination (administration of a non-absorbable antibiotic, rifaximin) or the use of TLR4 mutant mice showed, significant reduction of HSC activation, angiogenesis, portal hypertension and fibrosis[30].

Changes in TLR expression in response to acute or chronic stimuli are shown by parenchymal and non-parenchymal hepatic cells, as well as peripheral blood mononuclear cells (PBMCs). Although LPS and other TLR ligands can activate different signaling pathways in various cell types (immune and non-immune), promoting a proinflammatory and profibrogenic cascade in acute circumstances, anti-inflammatory and anti-fibrogenic mechanisms are present concurrently to balance these processes and maintain liver homeostasis and immunotolerance. The phenomenon of LPS hyporesponsiveness or LPS tolerance has been observed in monocytes, KCs and liver sinusoidal endothelial cells (LSEC) in response to repetitive stimulation with low dose of LPS. LPS tolerance accompanied by reduced nuclear translocation of nuclear factor (NF)-κB is caused by alterations in the TLR-4 signaling pathway. In LSECs, this process is associated with surface expression of CD54 or other leukocyte adhesion molecules and chemokines [*e.g.*, monocyte chemotactic protein-1 (MCP-1)], while in rest of the above-mentioned cell populations it is associated with decreased TLR-4 expression[31].

Functional impairment of TLR2 and TLR4, the most important PRRs for bacterial recognition, caused by sustained LPS exposure, appears to play a significant role in the risk of infection in cirrhotic patients[32]. Studies on PBMCs collected from patients with cirrhosis clearly showed that there was dampened TLR2 function, even in the early stage of cirrhosis[33,34]. Moreover, at least in advanced cirrhosis, TLR4 impairment was also present[33,35-38], where TLR function was assessed by TNF-α production in culture. Antibiotic or probiotic treatment was able to relieve the TLR disruption by increasing TLR4 levels and restoring receptor function[35,38]. It must be noted, however, that there are also some contradictory results, probably reflecting the heterogeneity of the patient population and methodological differences. Decreased TLR levels are not sufficient to alter the TLR function, which also suggests probable intracellular dysfunction[32].

### Functional polymorphisms of PRR

Inherited variations of PRR gene functions have proven to underlie the risk of infection in cirrhosis. In a prospective study by Nischalke *et al*[39], a TLR2 GT microsatellite polymorphism and nucleotide-binding oligomerization domain (NOD) 2 variants were independent predictors of spontaneous bacterial peritonitis (SBP) (OR = 3.8, $P = 0.002$ and OR = 3.3, $P = 0.011$, respectively) in a multivariate analysis. Both the NOD2 variants[40] and the TLR2 microsatellite polymorphism[41] were associated with reduced levels of NF-κβ activation, suggesting a signaling defect *in vitro* and decreased release of pro-inflammatory cytokines, such as TNF-α, IL-12, IL-6 upon *in vitro* stimulation with bacterial lysates. Additionally, in a study by Bruns *et al*[42], patients carrying the *TLR2* polymorphism Arg753Gln (the GA genotype) had SBP more often than patients with the GG genotype (55.6% *vs* 18.2%, $P = 0.019$).

Genetic immune defects could also contribute to the high risk of systemic bacterial infections in cirrhosis beyond SBP. In a retrospective Spanish study[43], patients with ascites carrying the *TLR4* D299G polymorphism showed a trend towards a higher incidence of history of bacterial infections and a significantly higher number of infections per patient than wild-type patients. This single SNP has been shown to change the ligand-binding site of the receptor, because it is located close to the TLR-4-MD-2 binding areas[44] and is associated with blunted physiological response to LPS[45]. However, the functional impact of *TLR4* (D299G) polymorphisms on the LPS-induced cytokine response is controversial[46-48]. Mannose-binding lectin deficiency (MBL)[15] and haptoglobin (Hp) polymorphism type 1-1[49] have been found to confer a higher risk of systemic bacterial infections in patients with cirrhosis (OR = 2.14, $P = 0.04$ and OR = 2.74, $P = 0.015$, respectively) independently of disease severity. MBL, belonging to C-type lectin family, recog-

nizes surface carbohydrate sequences of a wide range of pathogens and stimulates direct opsonophagocytosis *via* the lectin pathway of the complement system. In case of MBL deficiency, both the recognition and the eradication of the pathogens are impaired. Hp is an acute phase plasma protein. Three phenotypes of the molecule exist, each with biologically important differences in their antioxidant, scavenging and immunomodulatory properties. These differences influence the course of subsequent inflammatory diseases. Hp1-1 has a weaker bacteriostatic effect than Hp2-2 and potentiates a Th2 immune response, thus predisposing subjects with Hp1-1 to develop bacterial infections. There is also a link between Hp polymorphisms and the body's iron store. Excessive iron accumulation has an adverse effect on immunity. Iron overload seems to exert a subtle effect on the immune system by altering the proliferation of T and B-lymphocytes. Furthermore, bacteria utilize the iron of the host organism as an important nutrient[50-52].

Though all the above-mentioned host genetic factors associated with significant ORs suggest an important role of single nucleotide polymorphisms (SNPs) in determining infection risk, a key question remaining is how these markers could be utilized in these clinical settings. Several points are worth considering. First, the frequencies of these polymorphisms in the population are relatively low (around 10%), limiting their efficacy as predictors. Second, ethnic and geographical differences in these functional polymorphisms exist. For example, the occurrence of the NOD2 risk alleles is highest in central Europe, but is absent from certain non-Caucasian populations, thus preventing their universal application[53]. One study[39] showed that the combination of the markers (simultaneous presence of both genetic variants, TLR2 GT microsatellite polymorphism and NOD2 risk variant) specifically improved the identification of patients with a high risk for SBP (OR = 11.3, $P < 0.001$). ORs of single clinical factors or laboratory markers were indeed inferior to ORs obtained using SNPs related to host immunity. In contrast, disease severity determined by a more complex way, using the Child-Pugh, score was superior to single SNPs to predict the infections, mainly in patients with advanced disease. However, this aspect was rarely examined in the above-mentioned studies. In one of our studies[49], the presence of the advanced disease (Child C) was associated with the highest risk of infection (HR = 4.43, $P < 0.001$) and was at least double the risk value of any other clinical or laboratory marker in a multivariate Cox regression model. The occurrence of the Child C disease stage was 29% in this population. There is no data regarding the added value of using host genetic risk factors to assess infection risk in combination with Child-Pugh stages. In earlier stages of the disease, combination of clinical score with genetic markers more likely enhances the risk assessment of the infections than in advanced stage of the disease. This approach could help to optimize patient care by identifying a high-risk population in which prophylactic antibiotic treatment might prevent SBP and other systemic infections and, therefore, mitigate the acute and chronic progression of the disease and prolong survival.

Functional genetic variations of PRRs associated with stronger pro-inflammatory response, however, might pave the way to progression from the chronic inflammatory state to the definite breakdown of the liver tissue, resulting in the development of cirrhosis. Support for this concept comes from the study of Brun *et al*[13]. The authors reported enhanced progression of fatty liver disease according to -159C/T promoter polymorphism in the CD14 gene. This polymorphism was proved to influence the transcriptional activity, thus determining the expression level of CD14. Subjects carrying the TT genotype had the most prominent elevation in CD14[54] and TNF-$\alpha$[13] levels. As previously mentioned, several hepatic cell populations involved in liver damage and fibrogenesis can directly respond to LPS. Thus, increased CD14 expression in patients carrying the TT genotype might enhance their sensitivity to intestinal LPS and so augment the pro-inflammatory responses and disease progression in obese subjects. Accordingly, the TT genotype distribution was significantly higher in non-alcoholic steatohepatitis (NASH) patients than in control subjects or non-alcoholic fatty liver disease patients[13]. In patients with chronic hepatitis C infection, the-399T/I *TLR4* polymorphism was one of seven SNPs that may predict the risk of cirrhosis (for CC genotype: OR = 3.11, $P < 0.001$), supposedly related to its functional impact on the LPS-induced cytokine response[55].

## MONOCYTES

Impaired monocyte function, including defects in chemotaxis, superoxide generation[56], phagocytosis and killing activity, as well as a decrease in the production of lysosomal enzymes, are well-known components of CAIDS[57-59]. Numerous studies have investigated the role of monocytes in liver inflammation and fibrosis extensively[60-63], along with their indispensable involvement in "cirrhosis associated immunological dissonance"[37] and its clinical manifestation of increased susceptibility to bacterial infections or in ACLF. Zimmermann *et al*[61] found a significant increase in circulating monocytes, with a shift towards non-classical $CD14^{+}CD16^{++}$ monocyte subset in CLD patients. This non-classical monocyte subset possesses pro-inflammatory and pro-fibrogenic potentials; moreover, they express higher levels of CXCR3, MHC-Ⅱ (HLA-DR), FcγRⅡ and IL-2R (CD25) than the classical $CD14^{++}CD16^{-}$ monocyte subset[62,63]. Chemokine-mediated recruitment, accumulation and activation of $CD14^{+}CD16^{++}$ cells in the liver, along with consequent direct HSC activation, also contribute to the ongoing fibrogenetic processes[61-63]. Novel findings from Seidler *et al*[64] indicated that sIL-2R (sCD25) might be a potential biomarker of immune cells', especially $CD14^{+}CD16^{++}$ monocytes', activation in CLD. Independently of the underlying etiology, significantly elevated serum sIL-2R lev-

els were observed in established cirrhosis compared with controls and non-cirrhotic patients. sIL-2R levels were also correlated positively with total monocyte counts and subsets or non-invasive markers of fibrosis, and were inversely correlated with parameters reflecting the biosynthetic capacity of the liver. It should be noted that sIL-2R levels are influenced by renal function. Monocytes from ascitic patients with alcoholic cirrhosis, especially a subgroup with elevated LBP levels indicating enhanced BT, showed higher expressions of TNF-α, HLA-DR and CD80. Norfloxacin treatment *via* intestinal decontamination, and the consequential decrease of circulating bacteria and bacterial products, could normalize the number of circulating monocytes along with reduction of TNF-α expression and activated phenotype in these patients[65]. Intestinal decontamination with antibiotics, therefore, should be considered as a therapeutic weapon in restoring immune status and monocyte function in cirrhosis[66].

In contrast, functional monocyte deactivation, a phenomenon similar to *in vitro* LPS tolerance, is also described in patients with Child C cirrhosis and ACLF[36,37,67,68]. This phenomenon is presented as "immune paralysis" in the literature and is defined as downregulation of HLA-DR expression on monocytes. The etiological factor of "immune paralysis" was proven to be chronic endotoxemia by Lin *et al*[37]. Serum LPS levels correlated inversely with HLA-DR expression and positively with serum IL-10 levels, an anti-inflammatory cytokine. Supporting this observation, *in vitro* stimulation with LPS was able to suppress HLA-DR expression in monocytes derived from healthy volunteers in an IL-10-dependent manner. Monocytes from cirrhotic patients expressing low levels of HLA-DR showed a decreased ability to secrete TNF-α, accompanied by decreased expression of inducible nitric oxide synthase (iNOS) and co-stimulatory molecules (CD40, CD86). Furthermore, reduction in HLA-DR expression (< 40%) was associated with poor outcome in patients with ACLF[36,67], especially if monocytes were unable to show improvement in HLA-DR expression. The overall prognostic power, however, remains inferior to conventional markers. The sensitivity and specificity of reduced HLA-DR expression (< 40%) to predict the 90-d mortality were 59% and 80%, respectively[69]. In conclusion, "immune paralysis" is characterized by dominance of anti-inflammatory (elevated serum IL-6 and IL-10 levels) and suppression of pro-inflammatory processes (decreased TNF-α levels)[36,37,67,68]. In sepsis patients with reduced monocyte HLA-DR expression, the function of these cells could be restored with immunomodulatory agents like granulocyte-monocyte colony-stimulating factor (GM-CSF) and IFN-γ, thus their effect on monocyte function should be investigated in cirrhosis and ACLF[67].

## MACROPHAGES

The resident macrophages in the liver are the KCs and account for approximately 80% of all macrophages in the body[70]. At the same time, KCs are the second most abundant non-parenchymal cell type populating liver tissue after LSEC[11]. Three major pathogenetic roles of KCs are relevant to cirrhosis: (1) as the main orchestrating immune cells in the liver; the KCs and their cross talk with HSCs, the ultimate effectors of fibrogenesis in the liver, are in the focus of attention for understanding fibrogenetic mechanisms. Activation of KCs by PAMPs or DAMPs *via* PRR signaling pathways results in activation of HSCs and recruitment of phagocytic cells through secretion of proinflammatory cytokines, chemokines (*i.e.*, MCP-1) and upregulation of adhesion molecules, thus contributing to fibrogenetic processes[71]; (2) in addition, activated KCs, along with recruited bone marrow (BM)-derived macrophages through production of vasoconstrictor agents like thromboxane A2 ($TXA_2$), seem to increase portal pressure in normal and in fibrotic animal models[72]. This concept is supported by recently published studies, which found a strong correlation between sCD163, a biomarker of macrophage activation, and the hepatic venous pressure gradient (HVPG)[14,73-75]; and (3) additionally, the deficient phagocytic capacity of KCs in advanced cirrhosis can also eventually lead to decreased elimination of blood-borne pathogens and mainly intestine-derived bacterial products, thereby contributing to an increased risk of bacterial infection[76].

Recently, CD163 has been proposed to be a specific marker of monocyte/macrophage cell populations[77]. The utility of this SR is not yet fully understood, but it supposedly functions as an innate immune sensor for bacteria[78] and has an essential role in the inflammatory processes. During the local activation of macrophages, the extracellular portion of CD163 is cleaved by metalloproteinases and enters the circulation as a soluble protein (sCD163)[79]. It is now evident that sCD163 is very useful as a biomarker of macrophage activation in various inflammatory diseases, as well as in chronic liver diseases. An elevated sCD163 level is related to portal hypertension, indicated by HVPG value in patients with cirrhosis[75]. In a very recent prospective clinical study by Waidmann *et al*[14] high sCD163 levels were shown to be associated with both the development of variceal bleeding and mortality in cirrhosis, independently of endoscopic risk factors and the disease severity, respectively.

MCP-1 is one of the most potent chemokines for monocytes/macrophages and activated lymphocytes during infections[80]. MCP-1 also plays a role in the recruitment and maintenance of the inflammatory infiltrate during liver injury[81]. Similar to *PRR* genes, a functional polymorphism of *MCP-1* gene (-2518 G/A) can also influence both the risk of bacterial infections and hepatic inflammation and fibrosis progression. In a small study by Gäbele *et al*[82], the 2518 MCP-1 genotype AA was identified as a risk factor for SBP in patients with alcoholic cirrhosis, supposedly caused by reduced MCP-1 protein level in ascites. Evaluating HCV patients, Mühlbauer *et al*[83] reported that carriers of the G allele were significantly more frequent among patients with more

advanced fibrosis and severe inflammation. In support of this, hepatic MCP-1 mRNA levels and cytokine-induced MCP-1 secretion of isolated HSC were significantly higher in patients carrying the G allele. Furthermore, there was a binding activity in nuclear extracts from activated HSCs specifically to the G allele, providing a potential mechanism for the differences observed.

The liver, *inter alia*, functions as a bacterial filter and the sinusoidal KCs play an important role in the elimination of intestinal bacteria and endotoxins translocated from the intestine. Patients with cirrhosis have impaired function of the reticuloendothelial system (RES), along with a decrease in the number and function of KCs[76,84]. Additionally, because of the formation of collateral circulation, a certain proportion of the blood-volume bypasses the liver, reaching the systemic circulation directly. Although limited data is available regarding RES dysfunction, Rimola *et al*[76] found that patients with decreased RES phagocytic activity developed bacterial infections more frequently compared with patients with normal RES function. Dysfunction of KCs was also proven in new studies with superparamagnetic iron oxide-magnetic resonance image (SPIO-MRI) in NASH and cirrhosis[85,86]. Furthermore, impairment of Fcγ-receptor function and consequential decrease in clearance capacity in macrophages also contributes to an increased incidence of bacterial infections in cirrhosis[87].

## NEUTROPHILS

Polymorphonuclear leukocytes (PMNs) are present in a fully activated state in the peripheral blood in cirrhosis and alcoholic hepatitis, possibly because of sustained exposure to bacterial products, such as endotoxins[88]. This results in an energy depleted status of the PMNs, which have an inability to function properly (decreased chemotaxis, phagocytosis and bactericidal capacity)[66,89,90]. Removal of endotoxins *in vitro*[91] as well as attenuation of endotoxemia *in vivo* with probiotic[38] treatment can restore PMN function in cirrhosis, further supporting this hypothesis. Increased priming[92] and therefore "ready to act" status of PMNs is indicated by decreased L-selectin levels, overexpression of hydrogen peroxide and increased levels of neutrophil elastase)[93]. As a result of this preparedness to defeat bacteria and PMN activation with high resting respiratory burst activity[94], there is an elevation in harmful reactive oxygen species (ROS) in the circulation and the PMNs' microenvironment, establishing a platform for further potential cell and tissue injury. Necessarily, PMNs become energy depleted and unable to respond properly to further bacterial stimuli with phagocytosis[66,89]. Impaired tuftsin activity[95], hyponatremia and hyperammonemia[96,97], along with inadequate generation of superoxide anion caused by deficient phospholipase C (PLC) activity[98], all contribute to the aforementioned decrease of PMNs' phagocytic capacity. Elevated resting oxidative burst and the decreased phagocytic capacity appeared to correlate with the rate of infections and mortality[91]. These alterations can be restored *in vitro* by endotoxin removal[91] or GM-CSF incubation[99]. Analogous to other innate immune cells, dichotomy in PMN function (hyperactivity then dysfunction) manifests in different ways and contributes to the pathogenic processes in the distinct stages of cirrhosis. Recruitment of hyperactive PMNs to the liver can contribute to fibrogenesis, while exhausted PMNs defective in chemoattraction, enhanced adhesion to endothelial cells and deficient migration in later stage of cirrhosis can result in deficient influx into infected sites[90,100]. An *in vitro* study in cirrhotic patients demonstrated that G-CSF could enhance neutrophil transendothelial migration, despite having no effect on enhanced neutrophil adhesion[100]. Notably, in a randomized clinical trial, administration of G-CSF improved survival of patients with ACLF, partially through restoring PMN dysfunction. Though the exact mechanism of G-CSF improvement of PMN function has not yet been determined, increases in PMN surface antigen CD11b/CD18 expression, along with elevated plasma elastase-α1AT complex levels, were previously detected following G-CSF administration[101]. Apart from various functional impairments of PMNs, a decrease in cell volume as a result of hyponatremia and hyperammonemia[96,97] with reduced cell number (neutropenia) as a consequence of hypersplenia and shortened neutrophil survival *via* apoptosis[102], are also known features of CAIDS. The epidemiology[103], pathogenesis and clinical consequences of cirrhosis-associated neutropenia were reviewed in a recent publication by Kalambokis *et al*[104].

Genetically determined enhanced myeloperoxidase (MPO) activity caused by an SNP in the promoter region of the enzyme (G-463-A MPO polymorphism) in patients with GG-MPO genotype was found to be independently associated with increased risk of hepatocellular carcinoma (HCC) and liver-related death with or without HCC in alcoholic cirrhosis (HR = 4.7 and 3.6, respectively, $P < 0.001$ for both)[105]. Activated KCs and liver-infiltrating neutrophils release MPO into the extracellular space and mediate oxidative processes by hypochlorus acid[106].

## COMPLEMENT SYSTEM

Low opsonic activity and decreased complement levels, mainly C3, weaken the bacterial recognition and bactericidal capacity in cirrhosis[107,108], further contributing to an increased susceptibility to bacterial infections. One interesting feature of bacterial infections in the cirrhotic patient population is the extreme sensitivity to *Pneumococcus* pneumonia and the high mortality. The defect in early bactericidal activity of alveolar lining components (reduced levels of lysozyme and complement C3) is a probable explanation[109]. Overall, bacterial pneumonias are the third most frequent infections in cirrhosis, and comprise 15% of all systemic infections. In addition, the mortality rate of pneumonia is much higher than

that in any non-cirrhotic population[110]. Data concerning alterations of the lectin pathway of the complement system and their effect on the susceptibility to bacterial infection are scarce. Our group reported that MBL levels were significantly reduced in patients with the most advanced stages of cirrhosis and absolute MBL deficiency (< 100 ng/mL) was associated with higher probability and shorter time to develop bacterial infections in cirrhosis[15]. MBL antigen levels in the sera, estimated by a mannan-binding assay or complement activation in the C4b deposition assay, accurately indicated the function[111]. The serum levels of functional MBL also correlate well with underlying MBL2 genotypes. In this regard, other components of this third arm of the complement system (ficolins or MBL-associated serine protease-2) have not yet been studied.

## ADAPTIVE IMMUNE DYSFUNCTION

### *B-cells and immunoglobulins*

A broad defect of B-cells in patients with ALD and its association with the exposure to circulating antigens as a consequence of shunting, or KC abnormality, or both, has been known for a long time[112]. A very recent study of Doi *et al*[113] revealed novel information about the nature of the profound abnormalities in peripheral B-cell phenotype and function. B-cell dysfunction strongly implicated hepatic fibrosis and/or portal hypertension in the development of this phenotype, and it was independent of the etiology of the cirrhosis. Moreover, this study highlighted how these B-cell defects could explain, in part, the vaccine hyporesponsiveness and susceptibility to bacterial infection in this population. B-cell phenotypes were assessed by flow cytometry. $CD27^+$ memory B-cells and, more specifically, $CD27^+IgM^+$ B cells, were found to be markedly less frequent in cirrhotic patients. The frequency of $CD27^+/CD19^+$ B cells strongly correlated with several laboratory parameters related to progressive liver disease. Previously, peripheral B-cell CD27 expression was reported to be related directly to the capacity of B-cells activation by CD40 plus TLR9 ligation[114]. Accordingly, using isolated peripheral blood cells, the authors proved that B-cells were hyporesponsive to CD40/TLR9 activation, indicated by significantly reduced CD70 upregulation, less TNF-β secretion and IgG production. The allostimulatory capacity of cirrhotic B-cells on $CD4^+$ T-cell proliferation was also diminished. The presence of bacterial products in the circulation playing fundamental roles in driving B-cell changes in cirrhosis has been proposed. Soluble factors associated with BT, such as LPS[115,116] and bacterial DNA[117], can often be detected in cirrhotic plasma and are capable of activating B-cells *in vitro*. As a proof, Doi *et al*[113] found that blockade of TLR4 and TLR9 signaling abrogated the activation of healthy donor B-cells by cirrhotic plasma. The fate of lost $CD27^+$ B-cells remains incompletely defined.

Stimulation of B-cells by TLR ligands can lead to polyclonal activation and Ig production. Notably, in humans, TLR-2, TLR-4 and TLR-8 are expressed strongly by monocytes/macrophages, but are expressed poorly by B-cells. In contrast, TLR-7 and TLR-9 are expressed mainly by B lymphocytes and plasmacytoid dendritic cells[118,119]. In cirrhosis, there is an enhanced serum IgA level, mainly in those with an etiology of ALD. However, the mechanisms leading to the increase of IgA levels are not fully understood[120]. Previously, it was attributed, at least partially, to a defective clearance of IgA and IgA-immune complexes *via* altered monocytes, Fc receptor expression, and subsequent defective Fcα receptor-triggered endocytosis[121]. For a long while, it was hypothesized that the increase in Ig synthesis in alcoholic cirrhosis might be associated with bacterial stimulation[112]. Several reports now support this hypothesis. Massonnet *et al*[122] found significantly enhanced absolute IgA production by TLR-9 ligand CpG-activated B-cells in alcoholic cirrhosis compared to healthy subjects, which correlated with their intrinsic ability to produce spontaneously more IgA than healthy subjects. Relative TLR-9 ligand CpG-induced IgA production by purified B-cells from alcoholic cirrhotic patients was, however, less prominent, which corresponded to the lower TLR-9 expression on their B-cells compared to B-cells from healthy subjects. Such downregulation of TLR-9 expression by B-cells has been reported after *in vitro* CpG treatment, suggesting that the decrease in TLR-9 expression by B-cells from patients suffering in alcoholic cirrhosis could reflect *in vivo* priming by bacterial DNA during sustained BT[123].

Concerning IgA production, cirrhosis has another characteristic feature, namely the increased occurrence of various antibodies against gut bacterial proteins[97,124-127] or host proteins having cross-reactive epitopes with bacterial constituents[120,128,129] in the sera of the patients. These specific antibodies are present mainly in those patients with advanced diseases and portal hypertension. Moreover, positivity for anti-*Saccharomyces cerevisiae* antibody (ASCA)[97] was an independent risk factor for the development of clinically significant bacterial infections (OR = 1.63, $P = 0.018$). Similarly, presence of anti-neutrophil cytoplasmic antibody (ANCA) IgA was identified as an independent predictor for a shorter time to develop an infectious complication in multivariate Cox-regression analysis (HR = 1.74, $P = 0.006$), suggesting that serological response to various microbial components might be the consequence of sustained exposure to microbial antigens[129]. In non-vasculitic disorders, the presence of ANCA has been considered a sign of immunological response to enteric bacterial antigens[130,131]. Pathogen-induced inflammation might result in enhanced presentation of self-antigens because of molecular mimicry and the known pathogenic feature of *Helicobacter pylori*-associated human autoimmune gastritis[132]. In autoimmune liver disorders, atypical perinuclear-ANCA (atypical P-ANCA) has been reported to be directed against human β tubulin isotype-5 (TBB-5)

and cross-react with the bacterial protein FtsZ because of their extraordinarily high structural homology[133]. In the development of the enhanced IgA production, not only systemic overproduction, but also a contribution by the gut mucosal compartment is very probable. The composition and extent of the bacterial load in the gut have a very clear effect on IgA production. Sustained exposure to bacterial antigens during BT derived from the mucosal compartment might play a central role in the enhanced IgA class antibody formation in cirrhosis. Determination of the ratio of IgA1 and IgA2 subtypes, and detection of the secretory component (SC) on IgA molecules in sera, can help identify the location of antibody formation (bone marrow or mucosal compartment). An increase in the proportion of IgA2 subtype and the presence of SC are concurrently considered as confirmatory evidence for the mucosal origin of IgA secretion[134,135]. The proportion of IgA2 is about 10% of total IgA in human sera, while IgA1 is 90%, and they largely exist in the monomeric(m) form. The proportion of SC-containing IgA antibodies from the total IgA pool is no more than 1%, because SC is attached to di- or polymeric IgA (pIgA) *via* its transport through the epithelial cells into the gut lumen or to other mucosal surfaces[136].

Thus, in a recent work by our group[129], a detailed characterization of IgA type ANCAs revealed that the proportion of the ANCA IgA2 subtype was markedly elevated (46%), and SCs were present in the majority of ANCA IgA positive samples (87%) of our patients with cirrhosis. Moreover, high levels of total serum sIgA in alcoholic cirrhosis were reported in a study by Pelletier *et al*[137]. Both studies support significant gut involvement in IgA production. IgA has traditionally been regarded as a non-inflammatory antibody. Serum IgA, however, potently triggers (pro)-inflammatory activity upon binding to the myeloid IgA receptor, FcαRI[138]. Whether the elevated IgA has any harmful effect on disease progression remains to be determined. Parallel to specific IgA overproduction, there is a diminished IgG production. The more severe the liver disease, the more subtle the decrease in the specific IgG level in patients with cirrhosis[129]. The alcoholic etiology has an obvious negative impact on specific IgG production. These alterations in the ANCA IgA and IgG response clearly reflect those tendencies known from vaccination studies in this patient population, and presumably reflect the impaired adaptive immune system in cirrhosis, mainly in the advanced stage, and the direct inhibitory effect of alcohol on T-cell-mediated immunity[66]. After pneumococcal vaccination, anti-PPS (pneumococcal polysaccharide) IgA antibody levels were significantly higher than in control subjects, whereas IgG levels were reduced[139]. Considerably lower immunogenicity and faster decline of specific, protective IgG responses were reported in individuals with cirrhosis, particularly in the alcohol-induced form, after hepatitis B vaccination compared with CLD[140]. Patients with compensated cirrhosis were five times more likely to respond to hepatitis A vaccination compared with cirrhotic patients in the decompensated stage[141].

### T cells

Different T cell populations could possess either pro-, anti-fibrogenetic or dual properties regarding their relationship with HSCs. Elevated numbers of $CD8^+$ T cells and the consequential decrease in the $CD4^+/CD8^+$ ratio was associated with promotion of fibrogenetic processes in mice and humans. IL-17 producing $CD4^+$ T cells (Th17), along with NKT cells, seemed to be involved in fibrosis; however, their role in fibrogenesis is cytokine profile-dependent. Production of IL-17, IL-4 and IL-13 is somewhat pro-fibrogenetic, while secretion of IFN-γ, TRAIL and IL-22 is anti-fibrogenetic. In contrast, regulatory T cells ($CD4^+$ $CD25^+$ forkhead box P3 [FoxP3]) in the close vicinity of HSCs *via* secretion of IL-10 represent anti-fibrogenetic properties[23].

Similar to the changes in B-cell function, broad defects of T cells were also reported in an early publication of Nouri-Aria *et al*[112]. A recent study by Márquez *et al*[142] depicted an intensive derangement of T cell compartments of the immune system in patients with cirrhosis. High antigen load as a consequence of enhanced BT, indicated by elevated LBP levels, can contribute to prolonged activation and subsequent "exhaustion" of T lymphocytes. Significant reduction in the total number of peripheral blood T cells ($CD3^+$ cells) was observed in cirrhotic patients with ascites. The proportions of activated $CD4^+$ T cells (indicated by expression of CD25 and CD122 antigens) and senescence $CD8^+$ T cells ($CD8^+CD45RO^+CD57^+$ cells) significantly increased. Additionally, the proportion of memory $CD4^+$ and $CD8^+$ populations expressing apoptosis markers ($CD95^+$) was also higher in cirrhotic patients compared with healthy controls. Increased proportion of regulatory T cells [$CD4^+$ $CD25^+$ forkhead box P3 (FoxP3)] was also observed, and a significant correlation was found with LBP levels. Downregulation of lymphocyte co-stimulatory molecules, such as CD28, was also detected. Therefore, it can be speculated that these changes in adaptive immunity could play a role in the immunosupression seen in cirrhosis, leading to increased susceptibility to bacterial infections.

## RISK ASSESSMENT OF CIRRHOSIS RELATED BACTERIAL INFECTIONS IN THE CLINICAL PRACTICE

Standard clinical factors, and serological and genetic markers associated with immune dysfunction in cirrhosis all have their potentials, but they also have limitations to predict bacterial translocation, infections and disease progression in cirrhosis. The biological pathways involved in these processes, however, are multiple. It is most likely that these markers will be used for effective

risk assessment in combination, providing complementary information, rather than used singly. Clinical factors are easily accessible without cost, but may change during the long natural history and in certain cases are subjective, suffering from recall bias and inaccuracy. Laboratory tests have several advantages over clinical factors, such as objectivity, consistency during the disease course (for serological markers only in definite clinical circumstances) and higher odds ratio. However, they are not always widely available, and their costs could represent a drawback. Prospective clinical studies must be initiated to build up and validate composite score (CS) for risk assessment covering clinical factors and biomarkers.

### ACLF

ACLF is an increasingly recognized entity encompassing an acute deterioration of liver function in patients with cirrhosis, which is usually associated with a hepatic or extrahepatic precipitating event and results in the failure of one or more organs and has high short-term mortality. During evolution of cirrhosis, this condition comprises a distinct clinical entity from acute decompensation (AD)[3]. The recently published CANONIC study[142] established diagnostic criteria for ACLF and provided valuable data about its development and progression. The occurrence of ACLF is not rare, with approximately one-third of AD being associated with ACLF. From the immunological aspect, inappropriate regulation of the host inflammatory response to injury and infection plays an important role in the development of the disease. Exaggerated pro- and anti-inflammatory responses and their imbalance relative to each other are hypothesized to be the most important determinants in the disorder. In cirrhosis, both the systemic inflammatory response and the compensatory anti-inflammatory response (CARS) are more pronounced compared with those in normal subjects. It is likely that those patients that do not resolve the CARS are the ones that have highest mortality rates. The state of unresolved CARS (the so-called prolonged "immunoparalysis" state) may predispose patients to acquire infection that would further aggravate a pro-inflammatory response, resulting in a vicious circle[3,143]. In this acute situation, the presence of bacterial infection and/or enhanced BT trigger quite different processes compared with those relevant to the chronic progression of liver disease. The development of ACLF and multiorgan failure is characterized by significant alteration in systemic and hepatic hemodynamics, and worsening of the liver and the other organs' functions[3].

## CONCLUSION

In cirrhosis, the precise exploration of immune dysfunction has resulted in a more accurate understanding of the processes, leading to recognition of the development of complications in both the acute and the chronic progression of the disease. Considering the significant role of BT and bacterial infections in these processes, recognition how the host defense mechanisms are disrupted against invading microorganisms is of distinct clinical relevance. Early and efficient assessment of immune dysfunction using methods routinely available can assist clinicians in everyday practical decision-making when establishing treatment and care strategies for the patients with end-stage liver disease. The biological pathways involved in hepatic fibrogenesis and bacterial infections are multiple, suggesting that this goal can only be achieved by applying combinations of different markers. In the clinical setting, the establishment and validation of a composite score comprising clinical, serological and genetic markers could help to identify efficiently those patients at high-risk for progression and development of bacterial infections, even at an early disease stage. This would therefore lead to a decreased risk of complications, delayed progression of the disease and reduced mortality. Individually tailored steps for prophylaxis will enable clinicians to optimize patient care and expenditure.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

# Spinal cord involvement in patients with cirrhosis

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## Abstract

A severe spinal cord involvement may rarely occur in patients with cirrhosis and other chronic liver diseases; this complication is usually associated with overt liver failure and surgical or spontaneous porto-systemic shunt. Hepatic myelopathy (HM) is characterized by progressive weakness and spasticity of the lower extremities, while sensory and sphincter disturbances have rarely been described and are usually less important. The diagnosis is assigned in the appropriate clinical setting on clinical grounds after the exclusion of other clinical entities leading to spastic paraparesis. Magnetic resonance imaging is often unremarkable; however, also intracerebral corticospinal tract abnormalities have been reported recently. The study of motor evoked potentials may disclose central conduction abnormalities even before HM is clinically manifest. HM responds poorly to blood ammonia-lowering and other conservative medical therapy. Liver transplantation represents a potentially definitive treatment for HM in patients with decompensated cirrhosis of Child-Pugh B and C grades. Other surgical treatment options in HM include surgical ligation, shunt reduction, or occlusion by interventional procedures.



**Key words:** Hepatic myelopathy; Spastic paraparesis; Cirrhosis; Chronic liver disease; Porto-systemic shunt; Liver transplantation; Endovascular procedures

**Core tip:** This review article is worth reporting, because it provides a comprehensive and updated review of the most pathophysiological, clinical and therapeutical aspects of the hepatic myelopathy.

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## INTRODUCTION

Patients with chronic liver disease frequently experience neurological problems, with hepatic encephalopathy (HE) being the most common. Comparatively rare is the involvement of the spinal cord; the so-called hepatic myelopathy (HM) is usually associated with an extensive portosystemic shunt (PSS) of blood, either surgically created or occurring spontaneously.

In this review we will focus on the studies that have investigated the pathophysiology and the therapeutic strategies of this important, but likely often overlooked, neurological complication of chronic liver diseases. The MEDLINE, accessed by Pubmed (1966-August 2013) and EMBASE (1980-August 2013) electronic databases were searched using the medical subject headings (MeSH) "hepatic myelopathy", "liver cirrhosis", "spastic paraparesis", "chronic liver disease", "therapy", "liver transplantation".

Two review authors (YH and SL) screened the titles and abstracts of the initially identified studies to determine if they satisfied the selection criteria. Any disagreement was resolved through consensus. Full-text articles were retrieved for the selected titles, and reference lists of the retrieved articles were searched for additional publications.

The two reviewers independently assessed the methodological quality of each study and risk of bias, focusing on blinding and other potential sources of bias. The search strategy described above yielded 44 results. We excluded 2 studies after reading the full published papers; thus, 42 studies contributed to this review: the earliest was published in 1949 and the most recent in 2013.

## CLINICAL FINDINGS AND EPIDEMIOLOGY

Clinically, HM presents with an insidious onset of spasticity and weakness in the lower extremities, which slowly progresses over several years (decades) and causes the patients to become wheelchair-bound. Clinical findings of spastic paraparesis, usually without any motor deficits rostral to the cervical spinal cord segments, with hyperreflexia, extensor plantar responses and a puppet-like gait are characteristic of HM[1-18].

The most characteristic and distinctive feature in HM is a progressive lower extremity corticospinal tract deficit. Involvement of the upper extremities has rarely been described[3,19].

There are only a few reports of sensory or sphincter impairment[1,4,9,20]. Moreover, a delayed onset posterior column dysfunction (proprioception and vibratory sensory loss) and a small fiber length-dependent axonal polyneuropathy has been recently documented[16], both progressing concomitantly with the motor deficits. Most HM patients display normal or minimal sensory findings[5], but some patients exhibit more significant sensory deficits[1,4,9].

Since the first description of HM in 1949, there have been approximately 90 cases reported in the literature[14-18,21-32]. In rare instances, HM may be a presenting sign of liver disease[17]: Ben Amor *et al*[18] recently reported two patients who had no history of previous liver cirrhosis. In most of the reported cases, episodes of overt HE have preceded the development of the myelopathy[4,8,10,11]. HM can occur before or after HE, but also patients without any episodes of HE have been reported[18]. In the vast majority of the reported cases, the patients were males in the 5$^{th}$ decade of life at the time of their presentation with HM[14]. The middle age of onset is reported as 47 years.

The first reports of HM patients occurred when surgical shunting was more commonly performed. Some authors[11] have hypothesized that the incidence would eventually decrease as shunting became replaced by other less invasive treatments. However, with the transjugular intrahepatic portosystemic shunt (TIPS) becoming the standard procedure for refractory variceal bleeding, increasing reports of HM have emerged[15]. To date, there has been one case report describing reversal of HM by occlusion of TIPS[15].

## HISTOLOGY

The histology of HM consists of symmetrical loss of myelin in the lateral pyramidal tracts, with demyelination beginning in the cervical spine, becoming more intense at lower levels, and occasionally being associated with axonal loss[5,33]. In the early stages, demyelination seems to predominate, but as the disease progresses, axonal loss occurs, and this is likely to be irreversible[4,11]. Occasionally, demyelination has also been found in the ventral pyramidal tracts, in the posterior columns and spinocerebellar tracts. These pathological findings in the posterior columns of patients with HM were first described by Leigh *et al*[20] in 1949 and by Mendoza *et al*[11] in 1994. These findings raise the possibility that posterior column spinal cord pathology may be more common in HM than previously realized. Even if the lesions have shown up typically within the spinal cord, there are occasional reports of lesions within the brainstem without involvement of other tracts[20]. Additionally, Alzheimer type-Ⅱ cells and spongiform degeneration in the cerebral cortex have been described in HM[22].

## PATHOPHYSIOLOGY

The pathophysiology of HM is not yet completely understood. There is a close relationship between an extensive PSS and the occurrence of HM, even in the absence of liver dysfunction[10]. This observation supports the hypothesis that the shunting of blood may allow nitrogenous breakdown products or a neurotoxin to bypass the liver and damage the spinal cord. In particular, nitrogenous products such as ammonia have been identified as a major contributor to the development of HM[14]. Portocaval shunts or less commonly spleno-renal shunts seem to play a substantial role in the occurrence of HM-associated neurologic disturbances[34]. Shunts can occur spontaneously, after surgery, or due to "functional shunting" filtration of portal blood through a dysfunctional liver[14].

Impairment of neurological function in the form of encephalopathy was recognized in the early 20$^{th}$ century in patients undergoing surgical shunting or porticaval anastomosis (PCA) and was later described as "portal-systemic encephalopathy" by Sherlock *et al*[35]. Because some of the earliest reported patients with HM had undergone PCA, shunting was considered[36] a possible

explanation for the development of myelopathy, and a similar mechanism causing both HE and HM has been postulated. However, in most of the reported cases, episodes of overt HE have preceded the development of the myelopathy[33]. It has been suggested that a nutritional deficiency may underlie HM as a result of dietary restriction in patients with precedent episodes of HM[11]. However, this hypothesis is unlikely, because there are also reports of patients in whom HE never occurred[33] and who had been following a normal diet. Moreover, in contrast to HE, HM usually do not respond to blood ammonia-lowering therapies[26]. Therefore, the pathophysiology is most likely different in HM and HE. Protein restriction, as well as the use of lactulose and neomycin treatments, were not found to be beneficial for HM[1,5-7,10]. Moreover, surgical colonic diversion is helpful for HE, but does not reverse HM[37]. Treatments with lactulose, xifaxan, gabapentin, and pentoxifylline were also attempted in the interesting case reported by Caldwell *et al*[16], and none of the treatments was successful. Moreover, none of the HM patients who had eventually underwent LT had any reported neurological improvement in response to standard medical therapies for PSE[26-29].

Reversal of HM by occlusion of TIPS, as reported by Conn *et al*[15], lends support to some mechanism inherent to the presence of PSS. Approximately 20% of the patients (3/15) in the recent review by Caldwell *et al*[16] had no demonstrable evidence of PSS *vs* 10% of HM patients previously reported in the literature[15].

In addition to the possibility of a putative neurotoxin causing HM in patients with PSS, other etiological factors should be considered, including nutritional deficiencies[14] and metabolic abnormalities. Nutritional deficiency was first considered by Leigh *et al*[20] as a possible cause of the permanent spinal cord abnormalities observed in their patients. Vitamin B12 deficiency was taken into consideration in the two gastrectomy patients who later developed HM. However, the hematological profiles and vitamin B12 levels in these individuals were normal[1]. Serum vitamin B12 levels were normal in previously published HM patients[24-29], as well as in the patient reported by Caldwell *et al*[16], in whom serum vitamin B12, folate, and methylmalonic acid levels were within normal limits.

It has been suggested that altered circulation could increase spinal cord susceptibility to injury in HM. This was discussed in the context of portal hypertension[26,38], perhaps occurring in individuals with anatomic variants.

The topography of the spinal cord lesions in HM suggests that HM may be related to hemodynamic factors, as the observed lesions are located just within those spinal segments that miss an extensive collateral circulation[38,39].

HM can occur in patients with congenital hepatic fibrosis[10] and with focal nodular hyperplasia[40], and this observation underscores the point that the severity of HM does not necessarily parallel the degree of hepatic dysfunction.

## DIAGNOSIS

Diagnosing HM is often difficult, but it can be achieved after the exclusion of other causes of spastic paraparesis in the appropriate clinical setting. A detailed history, along with an accurate neurological examination including appropriate neurophysiological tests and neuroimaging procedures, are of crucial importance for the early detection of the disease. Other myelopathies with normal spine imaging should be included in the neurological differential diagnosis. These are listed in the algorithm proposed by Caldwell *et al*[16]: metabolic/nutritional diseases (renal disease, vitamin B/E or copper deficiency, lathyrism); vascular events (arterovenous malformation, infarct, vasculitis), spirochetes (Lyme, syphilis) or fungal (Cryptococcus, Aspergillus) infections, postinfection (transverse myelitis), autoimmunity (systemic lupus erythematosus, sarcoidosis, Sjogren's), neoplasm (lymphoma, paraneoplastic syndrome), toxicity (chemotherapy, radiation), genetic factors (leukodystrophy, Friedrich's ataxia), and motor neuron disease (amyotrophic lateral sclerosis). Magnetic resonance imaging (MRI) of the entire spinal cord and, when indicated, the brain is essential in the evaluation of HM. Infectious myelopathies can be assessed by patient history, spinal fluid analysis, imaging procedures, and serologies/cultures[26]. Infectious etiologies to consider include human immunodeficiency virus, human T-lymphotropic virus type-1, syphilis, and Lyme disease. A demyelinating syndrome that was recently reported in a patient with hepatitis B virus (HBV) manifested as a recurrent transverse myelitis with paraparesis and urinary retention[41]. Similarly, hepatitis A has also been implicated as a possible etiology for transverse myelitis[42]. However, none of the hepatotropic viruses (hepatitis A virus, HBV) has been implicated in HM. In the review by Caldwell *et al*[16] regarding HM patients after liver transplantation (LT), all 3 HCV patients exhibited reversal of the myelopathy, despite persistent viremia[25,26].

A paraneoplastic syndrome is another possible differential diagnostic consideration in the workup of HM, even if it has not been reported in the literature. The liver explanted by the Caldwell *et al*[16] contained a 1-cm hepatocellular carcinoma (HCC). One of the 2 patients with HM from the group of Koo and colleagues[28] also had HCC, but they had not undergone LT. That patient was a 64-year-old man with a 2.5-cm HCC who had undergone successful radiofrequency ablation of the lesion but exhibited no clinical or electrophysiological improvement up to 16 mo after treatment. HCC has been associated with necrotizing myelopathy in one case report[43], but in the most recent comprehensive review of HM, none of the 61 patients had an underlying diagnosis of HCC[15].

Table 1 shows the differential diagnosis and Table 2 the recommended diagnostic evaluation of patients with HM.

## NEUROPHYSIOLOGICAL FINDINGS

To determine the frequency and gravity of HM, Nardone

**Table 1 Differential diagnosis**

| |
|---|
| Brain pathology |
| Demyelinating processes |
| Hydrocephalus |
| Parasagittal space-occupying lesion |
| Arnold-Chiari malformation |
| Other structural abnormalities at craniocervical junction |
| Spinal cord pathology |
| Compressive myelopathy: spondylogenic cervical > thoracical myelopathy |
| Vascular myelopathy: spinal cord infarction, bleeding, vasculitis |
| Spinal cord injuries |
| Genetic disorders: Hereditary spastic paraparesis, adrenoleukodystrophy (spinal forms), Freidrich's ataxia |
| Metabolic/Nutritional: Subacute combined sclerosis (vitamin B12 deficiency), vitamin E deficiency, copper deficiency, latyrism |
| Toxic myelopathy: Chemotherapy, Radiation |
| Neoplasms: Extramedullary or intramedullary tumors, metastatic lesions, lymphoma, paraneoplastic syndome |
| Myelitis: |
| Viral infections (Virus varicella Zoster, Epstein Barr Virus, Herpes simplex virus, Citomegalovirus, myelopathy associated with acquired immunodeficiency syndrome (AIDS), Human T-Lymphotropic virus (HTLV- Ⅰ)-associated myelopathy/Tropical spastic paraparesis); |
| Fungal infections (Criptococcus, Apergillus); |
| Sprirochetal infections (Lyme disease, Syphilis) |

**Table 2 Diagnostic evaluation of patients with hepatic myelopathy**

| |
|---|
| History |
| Subacute bilateral lower limbs weakness; puppet-like walk or inability to walk in the setting of a chronic liver disease |
| Neurological examination |
| Spastic paraparesis, no sensory level, hyperreflexia, extensor plantar responses |
| Neuroradiological examination |
| Contrast enhanced MRI or computed tomography myelogram of the entire spine to rule out compressive etiology. MRI may show FLAIR signal prolongation in subcortical corticospinal tracts; |
| Brain MRI to rule out demyelinating processes, hydrocephalus, parasagittal space-occupying lesion, Arnold-Chiari malformation and other structural abnormalities at craniocervical junction |
| Other diagnostic tools |
| Lumbar puncture - examination of cerebrospinal fluid to rule out spinal cord inflammation or if neuroimaging is unrevealing; |
| Motor evoked potentials may disclose central conduction abnormalities even before the myelopathy is clinically manifest |
| Evaluation of spontaneous shunt |
| Abdomen ultrasonography, computed tomography, MRI (if no shunt visible, no history of portosystemic shunt or transjugular intrahepatic portosystemic shunt) |

MRI: Magnetic resonance imaging.

*et al*[27] performed a study examining motor evoked potentials (MEP) elicited by transcranial magnetic stimulation in thirteen patients with liver cirrhosis associated with PSS.

The six patients with clinical signs of spinal cord involvement exhibited severe neurophysiological abnormalities, more precisely, a prolonged central motor conduction time (CMCT), whereas interestingly milder but unequivocal MEP abnormalities were found in four of the seven patients with normal clinical examination. These findings indicate that the electrophysiological evaluation of central motor conduction may disclose an impairment of the corticospinal pathways even before HM is clinically manifest. The clinical and neurophysiological features of patients with slight MEP abnormalities improved after LT, whereas the patients with a more advanced stage of disease (severe MEPs abnormalities) did not.

The findings of Nardone *et al*[27] and Utku *et al*[22] support the potential value of evaluating CMCT in the preclinical and early stages of HM. Patients who undergo transplantation with preclinical or early HM by MEP/CMCT appear to have a greater likelihood of recovery both clinically and electrophysiologically[27]. It is thus possible that MEP/CMCT have greater sensitivity in detecting preclinical or early HM and in assigning a prognosis for recovery after LT. Although a larger study comparing the sensitivity, specificity, and predictive value of MEP/CMCT has yet to be conducted, central motor system neurophysiological studies are an important consideration in the workup of patients with HM.

Utku *et al*[22] performed a MEP study in two patients and found an absence of cortical MEPs in both the lower and upper extremities, and normal MEP values with radicular magnetic stimulation, suggesting that the lesion was localized within the cervical levels of spinal cord.

However, they could not perform any neuropathological investigations to corroborate this diagnosis.

Nardone *et al*[27] found an abnormal CMCT to the lower lumbar spinal segments and a normal CMCT to the upper cervical spinal segments, thus supporting localization to the thoracic spinal cord. Additionally, a MEP study of HM patients from Seoul[28] indicated that the sites of higher vulnerability are located between the upper thoracic and lumbar spinal cord.

Further MEP studies may not only provide a means for an early diagnosis, but also shed light on the spinal topography of HM.

## NEUROIMAGING FINDINGS

Most case reports have not documented MRI abnormalities in the spinal cord. This suggests that MRI may be less sensitive than MEP/CMCT in the early detection of HM or that, to date, abnormal corticospinal tract signals on MRI may have been underappreciated.

Negative spinal cord MRI findings support HM in the differential diagnosis, because MRI is essential to rule out compression of the spinal cord or myelitis[31].

However, abnormal spinal cord and even brain MRI imaging has been reported in HM patients[19,44]. In particular, the MRI finding of intracerebral corticospinal tract abnormalities in a recently reported patient[16] suggests the occurrence of HM-related pathology above the level of the foramen magnum. In fact, an increased FLAIR signal in the subcortical white matter and subcortical spi-

nal tracts was reported. This is the first report of an abnormal MRI intracerebral corticospinal tract FLAIR signal in HM, and indicates that the pathology of HM may not be confined to the spinal cord or that it may be tied to preclinical PSE. A similar abnormal FLAIR signal has also been described in PSE and cirrhosis[45]. Hyperintensity of the putamen and globus pallidus on T1-weighted MRI, attributed to manganese deposition in these nuclei, is not unique to HM and has been noted in other patients with chronic liver failure[46,47]. Although not specific to HM, these radiological findings correlated with the clinical findings in that patient. Interestingly, the improvement in abnormal brain imaging findings parallels the clinical improvement in spastic paraparesis after LT.

## THERAPY

HM has a poor prognosis because of its progressive and irreversible nature. Today, no therapy for this disorder has been established. Conservative treatment strategies for HM include liver protection, neurotropic drugs, and measures to control blood ammonia concentration. However, as previously mentioned, HM responds poorly to conservative medical therapy[15,48]. In particular, in contrast to HE, HM usually does not respond to blood ammonia-lowering therapies[26].

Surgical treatment options in HM currently include LT, surgical ligation, shunt reduction, or occlusion by interventional procedures. Surgical ligation has been reported to be effective, but is only used occasionally[22].

### *Endovascular interventional procedures*

Interventional endovascular shunt occlusion has been commonly used to treat encephalopathy due to post-surgical shunt and post-TIPS[15,48]. By contrast, the usefulness of this technique for post-surgical shunt HM has not yet been determined.

Recently, Wang *et al*[17] first reported reversal of HM by occlusion of a surgical splenorenal shunt using an AVP. In this interesting case, an impaird gait and a progressive decline in mobility were observed 14 mo after surgical splenorenal shunt. The patient had no history of HE, and his laboratory findings showed no liver dysfunction (with the exception of an increase in his serum ammonia level). Therefore, occlusion of the splenorenal shunt represented an alternative therapeutic option, and the large splenorenal shunt was successfully occluded using an AVP. Other possible embolizing materials for the embolization of the PSS are coils, and a detachable balloon. AVP implantation for this patient was chosen due to the relatively large size of the surgical splenorenal shunt. Moreover, coil migration can occur when used in short shunt tracts[49-52]. AVPs were recently found to be effective for the occlusion of internal iliac arteries[51], the treatment of pulmonary arteriovenous malformations[52] and the occlusion of a splenorenal shunt arising after TIPS[50-53]. AVPs have an advantage over coils in that AVPs can be more precisely placed within the vessel and that they can be repositioned or removed, if necessary.

Following AVP embolization, a gradual improvement in leg strength and balance was observed; seven months after AVP embolization, the patient was able to walk 1 to 2 km aided by crutches, with only mild residual spasticity of the lower extremities.

After PSS embolization a sudden increase in portal pressure may constitute a severe complication, resulting in aggravation of esophageal varices or even development of new varices[15,54,55]. Therefore, embolization should be performed only in patients with absent or mild esophageal varices and without signs of hepatic failure (*i.e.*, ascites or jaundice)[56]. Moreover, routine periprocedural endoscopy is recommended to minimize the incidence of embolization-related complications. Wang *et al*[17] used an occlusion balloon catheter initially to occlude the surgical shunt. Because further monitoring of the patient over a few days revealed no evidence of induced varices or ascites, an AVP was used to enable closure of the shunt.

Thus, Wang *et al*[17] are the first to report a surgical shunt related-HM successfully embolized with an AVP, which resulted in an immediate improvement in intrahepatic portal perfusion, a normalization of blood ammonia, and a gradual improvement of HM-related symptoms. The authors were also able to document a temporary balloon occlusion of the surgical shunt prior to permanent embolization, which also may be used to predict clinical and laboratory improvement.

### *Liver transplantation*

Campellone *et al*[13] were the first to suggest the use of urgent LT for HM because of the progressive and irreversible nature of the disease.

Until the advent of LT, slow progression of spastic paraparesis over several years inevitably caused HM patients to become wheelchair bound. In the reviewed literature, nearly all patients with symptomatic HM who eventually underwent LT had severe paraparesis before the operation and required either a cane or a wheelchair[24,26,28,29].

LT appears to be the only promising effective treatment modality for HM, as supported by several previously published reports[16,25-30]. In particular, outcomes for those patients who had undergone LT sooner after being diagnosed with HM suggest a potential neurological benefit[16,26,57]. In the case reported by Counsell and Warlow[24], LT was performed at least 18 mo after the onset of the myelopathy, and there was no improvement. In fact, LT earlier during the clinical course of HM and/or in the absence of marked abnormalities in MEP/CMCT is important in achieving satisfactory reversal of the neurological motor deficit.

It should be considered that, in HM patients with established cirrhosis, the degree of spastic paraparesis and the risk of permanency are discordant with the Child-Pugh score.

Interestingly, Caldwell *et al*[16] introduced the first use of a Model for End-Stage Liver Disease (MELD) points for the condition of HM to enable early LT resulting in

the reversal of marked spastic paraparesis. The patient underwent LT approximately 1.5 years after being diagnosed with HM. In this case there was no overt HE. Both the spastic paraparesis and posterior column deficits rapidly and markedly improved within 3 mo after successful orthotopic LT. Expedited orthotopic LT may lead to a favorable neurological outcome after the granting of MELD exception points for HM as the primary indication for LT. Thus, the MELD system does not automatically prioritize these patients for LT, and submission of an appeal is necessary. Increased awareness will aid earlier diagnosis of HM, and because good neurological outcomes can be achieved by prompt LT, the transplant community should consider early and rapid transplant evaluation for those patients with HM. On the basis of their review, Caldwell *et al*[16] concluded that patients with HM should be prioritized for LT with the consideration of MELD exception points.

## CONCLUSION

HM should be always considered in the differential diagnosis in patients with spastic paraparesis in the setting of chronic liver disease and/or portosystemic shunt. The diagnosis of HM should be established as early as possible to enhance the chance of a complete recovery of the spinal cord. Importantly, MEP studies may be suitable for the early diagnosis of HM, even in patients with preclinical stages of the disease. Even if HM is thought to be related to the increased shunting of portal venous toxins to the systemic circulation, conservative therapies are, unlike for HE, usually inefficient.

An early diagnosis of HM should prompt recognition of predisposing factors such as PSS or TIPS, which can be considered for shunt occlusion by interventional procedures. However, in most cases, LT represents the only option for patients with HM. In particular, LT remains a potentially definitive treatment for HM in patients with decompensated cirrhosis of Child-Pugh B and C grades[16,26,57], while for patients with normal liver function or Child-Pugh A grade cirrhosis the choice of LT *vs* other treatments remains debatable[15,22]. In these patients, shunt occlusion may represent a suitable alternative therapy to LT, and occlusion can help to relieve shunt-induced HM symptoms. In fact, in the case described by Wang *et al*[17], a large surgical splenorenal shunt was successfully occluded using an AVP, which resulted in significant clinical improvement of the shunt-induced HM symptoms. This technique represents a viable alternative to surgery or coil embolization, although further research is necessary. In addition, trial balloon occlusion of the shunt prior to performing permanent embolization can be used to predict clinical and laboratory improvement.

In conclusion, HM is a rare cause of spastic paraparesis, but clinical history, along with appropriate laboratory, neurophysiological and neuroimaging findings, may allow an early diagnosis in patients with chronic liver diseases.

We provide a comprehensive and updated review of the most pathophysiological and clinical aspects of HM. Moreover, we also discussed the appropriate and effective treatments for this possibly underrecognized neurological complication of liver cirrhosis.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

# Cirrhosis and hepatopulmonary syndrome

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## Abstract

Hepatopulmonary syndrome (HPS) is characterized as a triad: liver disease, intrapulmonary vascular dilatation and arterial hypoxemia. HPS is reported to be present in 4% to 32% of adult patients with end-stage liver disease and in 9%-20% of children. The pathogenesis of HPS has not been clearly identified. Portal hypertension causes impairment in the perfusion of the bowel and increases the enteral translocation of Gram (-) bacteria and endotoxins. This stimulates the release of vasoactive mediators, such as tumor necrosis factor-alpha, heme oxygenase-derived carbon monoxide and nitric oxide. Genetic alterations have not been associated with this syndrome yet; however, cytokines and chemokines have been suggested to play a role. Recently, it was reported that cumulated monocytes lead to the activation of vascular endothelial growth factor-dependent signaling pathways and pulmonary angiogenesis, which plays an important role in HPS pathogenesis. At present, the most effective and only radical treatment is a liver transplant (LT). Cirrhotic patients who are on the waiting list for an LT have a shorter survival period if they develop HPS. Therefore, it is suggested that all cirrhotic cases should be followed closely for HPS and they should have priority in the waiting list.



**Key words:** Cirrhosis; Hepatopulmonary syndrome; Pathophysiology; Liver transplantation

**Core tip:** Hepatopulmonary syndrome (HPS) is an important complication of cirrhosis. HPS is a significant factor in dyspnea and cyanosis in cirrhotic cases. At present, the most effective and only radical treatment is a liver transplant. Cirrhotic patients who are on the waiting list for a liver transplant have a shorter survival period if they develop HPS. Therefore, it is suggested that all cirrhotic cases should be followed closely for HPS and they should have priority in the waiting list. This review aims to reevaluate the recent progress in the diagnosis, pathophysiology and treatment of HPS.

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## INTRODUCTION

Pulmonary complications related to chronic liver diseases are frequently observed. The two most significant complications among them are hepatopulmonary syndrome (HPS) and portopulmonary hypertension (POPH). HPS is observed more frequently than POPH in patients with chronic liver diseases. HPS has been reported to be present in 4% to 32% of adult patients with end-stage liver disease[1,2], and in 9%-20% of children[3-5].

Kennedy *et al*[6] first defined HPS in 1977. HPS is characterized as a triad: liver disease, intrapulmonary vascular dilatation and arterial hypoxemia[7]. Cirrhosis is the most common condition associated with HPS. The cause of liver disease leading to portal hypertension does not seem to affect the development of HPS. HPS has been reported in patients with prehepatic portal hypertension

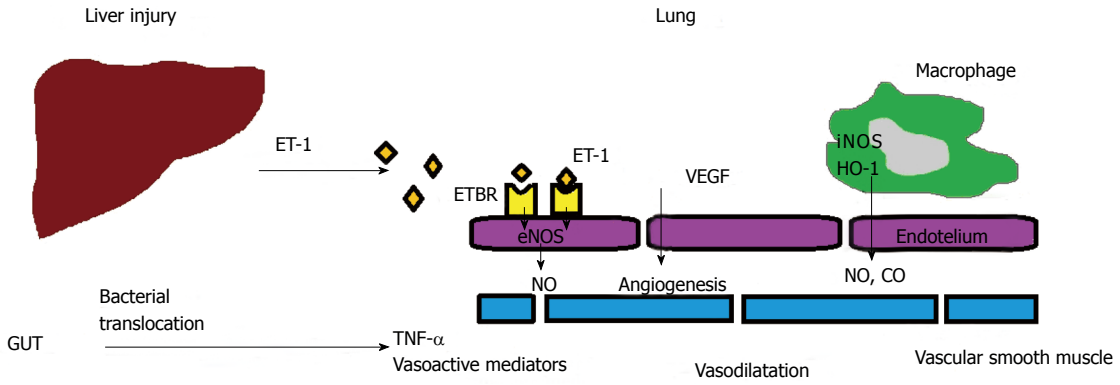


**Figure 1 Possible mechanisms of hepatopulmonary syndrome**. TNF-α: Tumor necrosis factor-alpha; ET-1: Endothelin-1; ETBR: Endothelin B receptors; NO: Nitric oxide; eNOS: Endothelial NO synthase; iNOS: inducible NO synthase; HO-1: Heme oxygenase-1; CO: Carbon monoxide; VEGF: Vascular endothelial growth factor.

in the absence of chronic liver disease, in Budd-Chiari syndrome and even in patients with acute or chronic inflammatory liver disease without evidence of cirrhosis or portal hypertension[8-12].

Prognosis is poor with the development of HPS in patients waiting for a liver transplant (LT). Therefore, these patients should be followed closely. This review aims to reevaluate the recent progress in the diagnosis, pathophysiology and treatment of HPS.

## PATHOPHYSIOLOGY

The pathogenesis of HPS has not been clearly identified. However, there are some important clinical clues. Although it has been observed in cases without cirrhosis and portal hypertension, most patients with HPS have cirrhosis and portal hypertension[4,13].

Portal hypertension causes impairment in the perfusion of the bowel and increases the enteral translocation of Gram (-) bacteria and endotoxins. This stimulates the release of vasoactive mediators, such as tumor necrosis factor-alpha (TNF-α), heme oxygenase (HO)-derived carbon monoxide (CO) and nitric oxide (NO)[14-18] (Figure 1).

In clinical studies, the increase of nitric oxide production in the lung plays a role in HPS pathogenesis[16-23]. When compared with cirrhotic control patients, exhalation NO levels increase in the cases with cirrhotic HPS. Plasma endothelin-1 (ET-1) levels increase in cases with cirrhosis and intrapulmonary vascular dilatation[24-26]. ET-1 causes NO-related vasodilatation with the activation of endothelin B receptors (ETBR) on endothelial cells[27,28]. In addition, increased phagocytosis of bacterial endotoxin in the lung promotes activation of inducible NO synthase (iNOS), which also contributes toward increased NO production. Bacterial translocation, and subsequent monocyte accumulation, may also stimulate pulmonary angiogenesis in HPS, which may partly be controlled by genetic factors[13].

Genetic alterations have not been associated with HPS yet; however, cytokines and chemokines have been suggested to play a role. It is more common in patients carrying the monocyte chemoattractant protein-1 (*MCP-1*) 2518G gene; conversely it is less frequent in patients with the endothelial NO synthase (eNOS) 298Asp allele and those carrying eNOS 298Asp[29]. These results suggest that the eNOS 298Asp polymorphism can prevent the development of HPS in cirrhotic patients. In addition, the G allele may be associated with higher MCP-1 expression in certain inflammatory conditions. The effect of the G allele appears to be dose dependent: cells from individuals homozygous for G at -2518 produced more MCP-1 than cells from G/A heterozygotes[30]. Higher levels of MCP-1, an inflammation marker, in HPS suggest the role of inflammation in the development of pulmonary shunts. Moreover, macrophages produce HO-1, which leads an increase in the production of CO and contributes to the vasodilatation[15]. CO is produced by the catabolism of heme with heme oxygenase[15,31].

Recently, cumulated monocytes have been observed to lead to the activation of vascular endothelial growth factor-dependent signaling pathways and pulmonary angiogenesis, which plays an important role in HPS pathogenesis[32]. Gene polymorphisms involved in the regulation of angiogenesis have also been associated with the risk of developing HPS[33].

Although other mediators, such as somatostatin analogue (octreotide), glucagon, prostacyclin, angiotensin-2, vasoactive intestinal peptide, calcitonin, substance P, atrial natriuretic factor and platelet-activating factor, may play a role in the pathogenesis of vascular changes in HPS, no clear relation was found between any of these mediators and vascular dilatation[34-39].

Important mechanisms mentioned above result in ventilation-perfusion (V/Q) mismatch, diffusion limitation of oxygen and, less commonly, direct arteriovenous connections[40,41]. The capillaries dilate to 15-500 μm (n: 8-15 μm) in HPS[42].

The hypoxia occurs because of the increased cardiac output caused by pulmonary vasodilatation and the inadequate oxygenation of the blood, which runs through

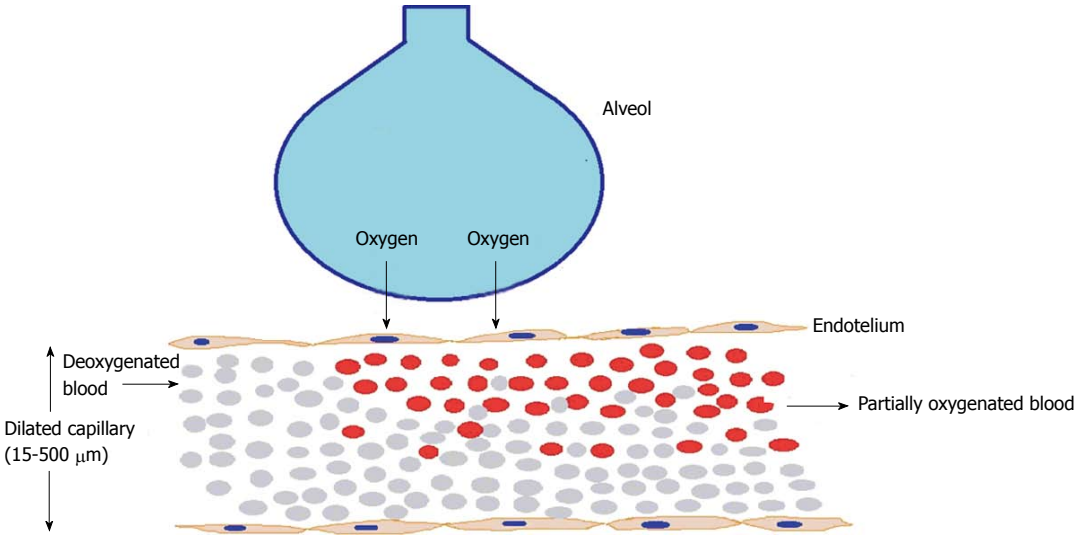


**Figure 2 Ventilation-perfusion mismatch; diffusion limitation of oxygen.** The capillaries are known to dilate to 15-500 μm (n: 8-15 μm) in hepatopulmonary syndrome (HPS).

enlarged pulmonary microcapillaries (Figure 2). In addition, hypoxia worsens as a result of the vasoconstrictor response to hypoxia, especially in the lower zones where there is less ventilation[43]. Lowered V/Q ratios result in elevated ΔP (A-a) $O_2$, which is correctable only by 100% oxygen inhalation. However, hypoxemia caused by larger arteriovenous shunts (AVS) does not respond to inhalation of 100% oxygen[44].

Previous studies have shown that certain changes occur in lungs of animals with HPS. Melo-Silva *et al*[45] showed that in rat models, the tidal volume, minute ventilation and mean inspiratory flow were significantly reduced, chest wall pressure dissipation against the resistive and viscoelastic components and elasticity were increased, and the lung resistive pressure dissipation was lower; however, the viscoelastic pressure was higher in the HPS group. The proportion of collagen volume in the vasculature increased by 29% in the HPS animals.

Furthermore, patients with hepatic cirrhosis have an elevated plasma level of lipopolysacchride (LPS)[46]. Extra LPS was given to rats with cirrhosis, which resulted in further widening of the alveoli wall, a decreased density of cells, narrowed alveoli space and destruction of the integrity of type I cell membrane, with infiltration of polymorphs and fibrinous exudates, indicating interstitial pulmonary edema and an inflammatory reaction. There was severe stasis of the blood in alveolar walls and numerous red cells extravasated the airspace, resulting in the widespread dilatation of alveolar capillaries and the augmentation of the permeability of the microvasculature[18].

## CLINICAL FEATURES

Cases of HPS can be asymptomatic or they can present with growth retardation, cyanoses, dyspnea, platypnea, orthopnea, spider angioma or finger clubbing. In a study conducted in The Mayo Clinic, 82% of 22 patients with HPS had symptoms and findings related to liver disease, and the time period from the respiratory complaints to the diagnosis of HPS was an average of 4.8 ± 2.5 years. In the same study, 18% of the patients complained of labored breathing and the liver disease was diagnosed after further tests[47]. The most frequent symptom was progressive dyspnea. Platypnea is defined as dyspnea of a patient when he/she is in an upright position. Digital clubbing and cyanosis are also frequent in HPS patients[29,48,49]. Spider angioma is not specific for the diagnosis of HPS[50]. The findings of chest radiography and decreased CO diffusion capacity determined by pulmonary function tests for the patients with HPS were nonspecific[51].

There is a poor correlation between liver disease and the level of oxygenation. While it has been reported mostly in patients with severely decompensated end-stage liver disease, namely child C patients, it has been also observed in patients with child A and child B cirrhosis[13,52].

In cirrhotic cases with suspicion of HPS, other cardiopulmonary diseases, such as pulmonary atelectasis, ascites, chronic obstructive pulmonary diseases, hepatic hydrothorax and infections should be ruled out.

Two types of HPS have been defined. While type 1 is related to precapillary dilatations, AVS is associated with type 2 HPS. In addition, while type 1 HPS responds to oxygen support, type 2 does not[53]. If $PO_2 \geqslant 600$ mmHg when the patient is given 100% oxygen, it is considered that it is probably not AVS. If the $PO_2$ fails rise to 150-200 mmHg or over, then the hypoxia may be considered to be caused by AVS[54].

## DIAGNOSIS

HPS is a serious complication frequently seen in cirrhotic patients and results from hypoxemia. The clinical findings in HPS are the same as those of hypoxemia. Contrast echocardiography (CEE) is accepted as a sen-

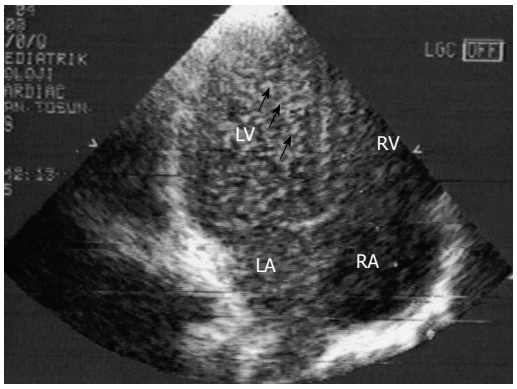


**Figure 3 "Bubble" or contrast echocardiogram (apical four chamber view).** A stream of microbubbles filling the left atrium following systemic venous injection is shown.

sitive screening test for HPS. Other techniques, such as technetium 99m-labeled macroaggregated albumin (MAA) scan, computed tomography or pulmonary angiography, have also been used to diagnose HPS[55]. Saline microbubbles are used for CCE. A transpulmonary passage was considered present if microbubbles appeared in the left atrium at least three heartbeats after the initial appearance of contrast in the right side of the heart[56-59] (Figure 3).

Transesophageal CEE increases the imaging quality of the heart; therefore, it is more sensitive than transthoracic CEE to identify intrapulmonary vasodilatation and may detect HPS, which can go unnoticed by transthoracic CEE[60]. For patients with hypoxemic HPS, there is no study that shows it is more superior. The detection of intrapulmonary shunts by this method is not sufficient to diagnose HPS and impaired oxygenation is required to be shown.

In addition, in cases with HPS, left ventricle enlargement and higher systolic velocity in the mitral valve represent satisfactory indirect markers of HPS[41], in addition to CEE[61].

Orthodeoxia, positional modification of abnormal shunting, was defined as a fall in $PaO_2 \geqslant 5\%$ when upright, or 4 mmHg. While the $PaO_2$ is normal in the horizontal position, it decreases in the upright position, depending on the increase of blood flow velocity in arteriovenous anastomosis in the basal segments of the lungs, caused by the effect of the gravity. This increases the ventilation-perfusion mismatch and hypoxia becomes apparent[62]. Orthodeoxia has been reported to be present in 20% to 80% of patients with HPS[63,64]. Hypoxemia is defined as $PaO_2 < 70$ mmHg, and severe hypoxemia is defined as $PaO_2 < 50$ mmHg. At sea level, and while breathing room air, a resting $PA\text{-}aO_2$ of $\geqslant 2.0$ kPa ($\geqslant 15$ mmHg) can be considered abnormal. For the people over 64, it should be evaluated as $PA\text{-}aO_2 \geqslant 20$ mmHg. Accordingly, the $PA\text{-}aO_2$ *vs* $PaO_2$ calculation is done to grade the severity of HPS[58] (Table 1).

In recent years, transcutaneous oxygen saturation measurement with pulse oximetry has emerged a simple, low cost, and widely available technique to screen for HPS. With a threshold value of < 96%, pulse oximetry has a sensitivity and specificity of 100% and 88%, respectively, for detecting patients with a $PaO_2 < 60$ mmHg. A pulse oximetry value of < 94% detected all patients with a $PaO_2 < 60$ mmHg with an increased specificity of 93%[65,66]. Contrary to these findings, CEE may be positive despite normal arterial blood gases. In a prospective study of candidates for LT, Krowka *et al*[67], found that 9.7% of 31 normoxemic patients had positive CEE. These findings suggested that mild or subclinical intrapulmonary vasodilatations insufficiency in cirrhotic patients may not alter gas exchange.

**Table 1 Grading of the severity of hepatopulmonary syndrome[58]**

| Stage | $PA\text{-}aO_2$ mmHg | $PaO_2$ mmHg |
|---|---|---|
| Mild | $\geqslant 15$ | $\geqslant 80$ |
| Moderate | $\geqslant 15$ | < 80 - $\geqslant 60$ |
| Severe | $\geqslant 15$ | < 60 - $\geqslant 50$ |
| Very severe | $\geqslant 15$ | < 50 (< 300 on 100% $O_2$) |

$PA\text{-}aO_2$: Alveolar-arterial oxygen tension difference; $PaO_2$: Arterial oxygen tension.

In the Technetium 99m-labeled MAA scan, MAA particles are given intravenously. The diameter of the marked particles is 20-50 μm and normally they cannot pass through pulmonary veins, which have a diameter of 8-15 μm. However, in the presence of an intrapulmonary shunt, these marked particles enter the circulatory system and appear in the kidneys and the brain. In the diagnosis of HPS, a value greater than 6% is significant and specific for HPS. However, as MAA provides positive results in the presence of intracardiac shunts as well; therefore, its sensitivity is low[41,68].

Unless there is an accompanying pulmonary disease, the spirometric tests in HPS are not impaired. However, abnormal diffusion capacity for carbon monoxide (DLCO) is frequently observed in patients with HPS[69]. In one study, the DLCO was decreased in 80% of the cases[70]. However, its specificity is low; therefore, it is not used in practice.

Pulmonary angiography is more invasive and less sensitive compared with high resolution chest computed tomography CEE[71] (Figures 4 and 5).

## TREATMENT

Currently, there are no effective medical therapies for HPS. In the past, HPS was considered as a contraindication for LT because of serious operative and perioperative complications in adults[72]. Today, LT is the only effective treatment option for patients with this condition, because of the underlying liver disease.

Although several investigations have been performed, no effective medical treatment has been found. Several attempts have been made to inhibit the development of HPS by administering nitric oxide, using diets low in

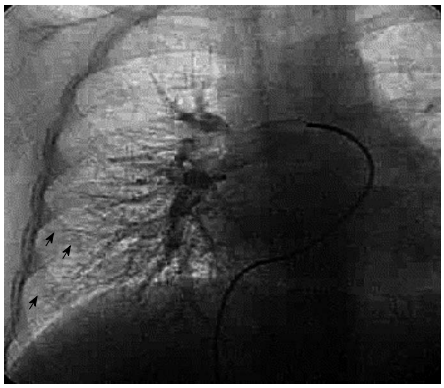

**Figure 4 Right pulmonary artery angiogram (posteroanterior projection) showing a diffuse fine reticular pattern of multiple pulmonary telangiectasias consistent with type I hepatopulmonary syndrome.**

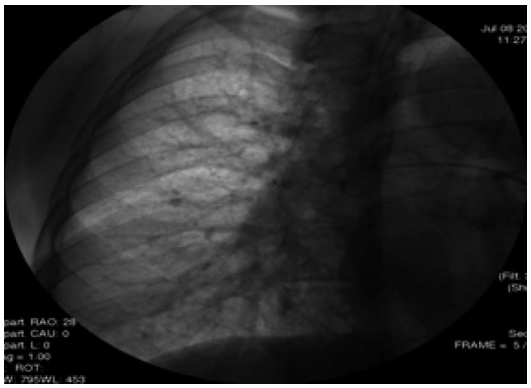

**Figure 5 Normal pulmonary angiography.**

L-arginine using methylene blue, which is an inhibitor of guanylate cyclase[73], aspirin[36], somatostatin[49,74], almitrine[75], N-acetylcysteine[76], indomethacin[51], garlic[77,78], mycophenolate mofetil (an inhibitor of angiogenesis and nitric oxide production)[79], pentoxifylline[80], decreasing the increased portal pressure by transjugular portosystemic shunt[81-83], and using antibiotics to decrease bacterial translocation in the bowel[84]. However, a role for any of these drugs in the long-term treatment of HPS has not been demonstrated. Currently, the most effective treatment is LT.

In cirrhotic cases with HPS, survival was significantly decreased compared with cirrhotic cases without HPS. In one study, the five-year survival rate was determined as 23% for cases with HPS and 67% for patients without HPS[85]. In studies performed at The Mayo Clinic, 33%-40% of patients with HPS died within 2.5-4 years. Most of the patients having clinically stable hepatic function worsened with the development of HPS[47]. In our center, eleven of 16 patients (68.7%) with HPS died before LT. The main causes of deaths were variceal bleeding accompanying multiorgan failure, pneumonia and sepsis[4].

Therefore, in addition to their Model for End-Stage Liver Disease (MELD) scores, it is very important for patients on the waiting list to be diagnosed as having HPS or not. However, many clinical features of HPS that might influence exception to the MELD scoring system, including standardized diagnostic criteria, pre- and post-LT mortality rates, and the rate of progression of hypoxemia, are not fully characterized[86]. In the United States, UNOS allows patients with confirmed HPS to be listed for an LT. For this reason, it is suggested that the patients with $PaO_2$ < 60 mmHg on room air in the sitting position, should receive an increase in their MELD scores, and during their waiting period they should get a 2- to 3-point increase in their MELD scores every three months[86]. The indications are that patients who had an LT this way showed better survival. Iyer *et al*[87] observed a trend (without reaching statistical significance) for better 5-year survival in the MELD exception era (since 2002) as compared to earlier HPS transplants.

The severity of preoperative hypoxemia, underlying liver disease and comorbidities appear to be factors that increase postoperative mortality[88,89]. While some studies show that the mortality rate of cases with HPS are 29%-38.5%[4,90], there are other studies that show the survival after an LT is not different to that od the cirrhotic cases without HPS[84,89]. In fact Iyer *et al*[87] suggested that survival post-LT was not dependent on baseline $PaO_2$ values obtained at the time of HPS diagnosis.

85%-100% of patients have an improvement in oxygenation within 1 year of an LT[4,53]. However, in some severe cases with hypoxemia, lung function may not recover up to one year after the operation. Most of these cases need oxygen for 5 to 700 d, and spend longer in hospital[89].

After liver transplantation and after all the factors that led to HPS have disappeared, patients recover from HPS[91,92]. In a single post mortem study, the changes after the transplantation in lungs in HPS could be related to collagen tissue deposition in pulmonary capillary and venule walls[93]. However, the recovery from HPS after transplantation shows that the pathological changes in the lungs in Type I HPS are reversible.

In our center, the perioperative and postoperative outcomes were uneventful in all patients. Even if previous reports indicated that patients with HPS require ventilatory support after an LT[67,94], two patients remained on mechanical ventilation and they were extubated two and five days post-transplant, respectively. If the postoperative hypoxemia is refractory to standard treatments, NO and trendelenburg positioning (supine position with feet elevated 15°-30° higher) and oscillator ventilation therapy are reported to be effective[89]. Experiences related with outcome of HPS after an LT are limited; portal venous thrombosis, intracranial events and multiorgan failure are seen more commonly in patients with HPS, and these complications are associated with a higher mortality rate[95]. In our series, we did not observe these complications, except for one case of acute respiratory distress syndrome, which was observed on postoperative day 1. This was probably related to systemic inflammatory response syndrome, because the patient had arterial

hypoxemia, fever and ground glass opacity on the chest radiography.

In conclusion, HPS is an important complication of cirrhosis, causing dyspnea and cyanosis in cirrhotic cases. There is no relation between the development of HPS and the severity of cirrhosis. At present, the most effective and only radical treatment is an LT. Cirrhotic patients who are on the waiting list for an LT have a shorter survival period if they develop HPS. Therefore, it is suggested that all cirrhotic cases should be followed closely for HPS and should have priority in the waiting list.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

# Management of thrombocytopenia due to liver cirrhosis: A review

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## Abstract

Thrombocytopenia is a common complication in liver disease and can adversely affect the treatment of liver cirrhosis, limiting the ability to administer therapy and delaying planned surgical/diagnostic procedures because of an increased risk of bleeding. Multiple factors, including splenic sequestration, reduced activity of the hematopoietic growth factor thrombopoietin, bone marrow suppression by chronic hepatitis C virus infection and anti-cancer agents, and antiviral treatment with interferon-based therapy, can contribute to the development of thrombocytopenia in cirrhotic patients. Of these factors, the major mechanisms for thrombocytopenia in liver cirrhosis are (1) platelet sequestration in the spleen; and (2) decreased production of thrombopoietin in the liver. Several treatment options, including platelet transfusion, interventional partial splenic embolization, and surgical splenectomy, are now available for severe thrombocytopenia in cirrhotic patients. Although thrombopoietin agonists and targeted agents are alternative tools for noninvasively treating thrombocytopenia due to liver cirrhosis, their ability to improve thrombocytopenia in cirrhotic patients is under investigation in clinical trials. In this review, we propose a treatment approach to thrombocytopenia according to our novel concept of splenic volume, and we describe the current management of thrombocytopenia due to liver cirrhosis.



**Key words:** Liver cirrhosis; Thrombocytopenia; Thrombopoietin; Partial splenic embolization; Splenectomy

**Core tip:** The major mechanisms for thrombocytopenia in liver cirrhosis are (1) platelet sequestration in the spleen; and (2) decreased production of thrombopoietin in the liver. For thrombocytopenia that is caused by platelet sequestration in the spleen, partial splenic embolization or laparoscopic splenectomy are effective. Thrombopoietin agonists and targeted agents are alternative tools for noninvasively treating thrombocytopenia due to decreased thrombopoietin production, although their ability to improve thrombocytopenia is under investigation in clinical trials. In this review, we describe the current management of thrombocytopenia due to liver cirrhosis, and we propose the novel concept of using the splenic volume to discern the primary cause of thrombocytopenia due to liver cirrhosis.

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## INTRODUCTION

Thrombocytopenia is a common complication in liver disease, and liver disease-related thrombocytopenia is often defined as a platelet count < $100 \times 10^9$/L, including moderate (less than $100 \times 10^9$/L) and severe (less than $50 \times 10^9$/L) thrombocytopenia. Although clinically significant spontaneous bleeding does not usually occur until the platelet count is less than $10 \times 10^9$/L-$20 \times 10^9$/L, cirrhotic patients with or without cancers often require numerous medical and/or surgical procedures during diagnosis and therapy. The presence of thrombocytopenia can aggravate surgical or traumatic bleeding and can also significantly complicate routine patient care, such as liver biopsy, antiviral therapy, and medically indicated or elective surgery for cirrhotic patients, resulting in delayed or cancelled medical management and affecting the administration of effective treatment for several conditions (*e.g.*, antiviral therapy for chronic hepatitis C virus (HCV) infection or cancer chemotherapy). Indeed, the degree of thrombocytopenia has been shown to be a useful prognostic marker in cirrhotic patients because the finding of severe thrombocytopenia (< $50 \times 10^9$/L) in liver disease can be associated with significant morbidity[1,2]. Additionally, a decreased platelet count can often be a diagnostic clue to unsuspected cirrhosis and to the presence of esophageal varices[3-6]. Multiple factors, including splenic sequestration, reduced activity of the hematopoietic growth factor thrombopoietin (TPO), cirrhotic coagulopathy, cirrhotic bone marrow suppression by chronic HCV infection and anti-cancer agents, and antiviral treatment with interferon (IFN)-based therapy, can contribute to the development of thrombocytopenia in cirrhotic patients. Of these factors, the major mechanisms for thrombocytopenia in liver cirrhosis are (1) platelet sequestration in the spleen; and (2) decreased production of TPO in the liver. Historically, thrombocytopenia has been thought to arise from the increased pooling of platelets in an enlarged spleen (splenomegaly). While the normal splenic volume has been reported to range from 50-200 mL[7], splenomegaly sometimes increases it to even more than 1000 mL. Platelet sequestration is seen in congestive splenomegaly due to cirrhosis-induced portal hypertension and is characterized by a redistribution of platelets from the circulating pool to the splenic pool[8]. However, the interventional and/or surgical treatments aimed at reversing portal hypertension do not always correct thrombocytopenia in the clinical setting. Indeed, decreased platelet production has been noted, even in patients without splenomegaly[9], suggesting that other factors are involved in thrombocytopenia due to liver cirrhosis. Platelets are derived from megakaryocytes, and TPO is known to be a potent cytokine that regulates megakaryocyte and platelet production[10,11]. TPO, which is primarily produced in the liver but is also produced, to a lesser extent, in the bone marrow and kidney[12,13], binds to the TPO receptor (c-Mpl), which is expressed on the surface of stem cells, megakaryocyte progenitor cells, megakaryocytes, and platelets. Experimentally, when TPO or its receptor (c-Mpl) has been "knocked-out" by homologous recombination in mice, the megakaryocyte and platelet masses are reduced to approximately 10% of the normal value, even though the animals are healthy and do not spontaneously bleed[14-16]. Cirrhotic patients with thrombocytopenia have lower circulating TPO levels than do cirrhotic patients with normal platelet counts, possibly as a result of diminished TPO production[17]. Interestingly, following successful liver transplantation or splenic embolization, the TPO levels appear to normalize, suggesting that decreased TPO production in the liver may also contribute to thrombocytopenia in cirrhotic patients[17-19]. This review describes the current management of thrombocytopenia in cirrhotic patients and also proposes a treatment approach for thrombocytopenia based on using the splenic volume to distinguish among the major causes of thrombocytopenia (splenic and other mechanisms, such as decreased TPO production).

## MANAGEMENT OF THROMBOCYTOPENIA DUE TO LIVER CIRRHOSIS

Several treatment options, including platelet transfusion, interventional splenic artery embolization, and surgical splenectomy, are now available for thrombocytopenia in cirrhotic patients. Therapeutic options to safely and effectively raise the platelet level can have significant effects on the care of cirrhotic patients. Specifically, an increase in platelet levels can significantly reduce the need for platelet transfusions and facilitate the use of IFN-based antiviral therapy and other medically indicated treatments in cirrhotic patients. Recently, treatments such as interventional management using partial splenic embolization (PSE) and surgical splenectomy have often attempted to correct splenomegaly-associated thrombocytopenia as the only current tool that noninvasively improves thrombocytopenia is the administration of platelet infusions. For example, antiviral therapy against HCV using peginterferon (Peg-IFN) alfa-2a plus ribavirin was discontinued in up to 2.6% of patients because of laboratory abnormalities, including thrombocytopenia, and 3%-5% of patients receiving Peg-IFN alfa-2a or alfa-2b plus ribavirin required dose modification because of thrombocytopenia[20,21]. In contrast, the management of thrombocytopenia using PSE or splenectomy prior to antiviral therapy successfully enables avoidance of discontinuation because of thrombocytopenia[22,23]. Recently, a low platelet count has been reported to be a predictor of liver atrophy and long-term mortality in patients on a liver transplant waiting list[24]. Additionally, experimental studies have shown that platelets can promote liver regeneration and improve liver fibrosis[25-27]. Indeed, PSE has a clinically improved prognostic outcome in cirrhotic patients[28]. Although invasive procedures such as PSE and splenectomy are occasionally underused due to potential interference with treatment options, including

**Table 1 Thrombopoietin-receptor agonists for the treatment of thrombocytopenia**

| Agent | Target disease | Dose (route) |
|---|---|---|
| Recombinant human thrombopoietin | | |
| rhTPO | Withdrawn from clinical use | (intravenous) |
| PEG-rHuMGDF | Withdrawn from clinical use | (subcutaneous) |
| rhIL-11 | Chemotherapy-induced TCP[40,42] | 50 μg/kg per day (subcutaneous) |
| | TCP in patients with cirrhosis[43] | 50 μg/kg per day (subcutaneous) |
| TPO mimetrics (peptide TPO receptor agonists and nonpeptide TPO receptor agonists) | | |
| Romiplostim | ITP[47-50] | 0.2-10 μg/kg once a week (subcutaneous) |
| | Myelodysplastic syndrome[51-53] | Once a week (subcutaneous) |
| | HCV-related TCP[54,97] | 2 μg/kg once a week (subcutaneous) |
| Eltrombopag | ITP[55,56] | 50 mg once daily (oral) |
| | HCV-related TCP | 25 mg once daily (oral) |
| E5501 | HCV-related TCP (phase Ⅱ; NCT00914927) | |

TPO: Thrombopoietin; PEG-rHuMGDF: Pegylated recombinant human megakaryocyte growth and development factor; rhIL-11: Recombinant human interleukin-11; TCP: Thrombocytopenia; ITP: Immune thrombocytopenic purpura; HCV: Hepatitis C virus.

liver transplantation, aggressive rather than passive management for thrombocytopenia may improve long-tern survival in cirrhotic patients. Although TPO agonists and targeted agents are alternative tools for noninvasively treating thrombocytopenia due to liver cirrhosis, their ability to improve thrombocytopenia in cirrhotic patients is under investigation in clinical trials.

### Platelet transfusion

Patients with platelet counts below $50 \times 10^9$/L may benefit from prophylactic transfusions to increase platelet counts before procedures. Guidelines for when to use platelet transfusions are available, but the relevance of these published guidelines for liver cirrhosis is unclear. The American Society of Clinical Oncology recommends platelet transfusions for cancer patients with platelet counts of $10 \times 10^9$/L-$20 \times 10^9$/L, depending on the type of cancer[29]. Currently, there is no consensus on the appropriate threshold values for prophylactic platelet transfusions in cirrhotic patients. Complications and limitations of platelet transfusion include febrile nonhemolytic and allergic reactions, the need for hospitalization, iron overload (with chronic transfusions), the risk of infection, platelet refractoriness due to HLA alloimmunization (occurring in up to 40% of patients), and cost[30,31]. Furthermore, platelet transfusions do not ensure a hemostatic platelet level, especially when the risk of bleeding is high[29]. While red blood cells have a lifespan of approximately 120 d, transfused platelets have a shorter life span and will need to be re-dosed within 3-4 d if given for prophylaxis.

### Agents targeting the TPO receptor

TPO plays an important role in regulating thrombopoiesis. The decrease in TPO production or activity in cirrhotic patients suggests that TPO can serve as a rational therapeutic target to stimulate platelet production. Several promising novel agents that stimulate TPO and increase platelet levels have been under development for the prevention and/or treatment of thrombocytopenia. Several types of TPO agonists and targeted agents, such as recombinant TPO, interleukin (IL)-11, and TPO mimetrics (peptide and nonpeptide TPO receptor agonists), have been evaluated (Table 1).

### Recombinant TPO and other thrombopoietic agents

Two forms of recombinant human TPO were evaluated in clinical trials and shown to increase megakaryopoiesis and thrombopoiesis: recombinant human TPO (rhTPO) and its pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF). rhTPO is a glycosylated form of TPO consisting of the full-length, native human amino acid sequence. rhTPO has been shown to be a potent stimulator of megakaryocyte growth and platelet production, and it is biologically active in reducing the thrombocytopenia of nonmyeloablative chemotherapy[11]. Although rhTPO could significantly increase platelet counts, the reduction of thrombocytopenia was not always accompanied by a decrease in transfusions[32]. In addition, rhTPO failed to demonstrate the clinical benefits of time to platelet recovery or platelet transfusion requirements in stem cell transplantation or leukemia chemotherapy[11]. Thus, the role of rhTPO in the treatment of thrombocytopenia was limited, and the clinical development of rhTPO was halted. PEG-rHuMGDF is an N-terminal TPO derivative that was pegylated to extend its half-life and retain its TPO activity[11,33]. In initial trials in patients undergoing chemotherapy, PEG-rHuMGDF treatment increased the median platelet nadir counts and enhanced recovery in a dose-dependent manner[34-36]. However, some subjects, including normal platelet donors treated with PEG-rHu-MGDF, developed neutralizing antibodies that cross-reacted with and inactivated endogenous TPO, resulting in extremely severe thrombocytopenia[32,37,38]. The clinical development of PEG-rHuMGDF by Amgen was stopped in 1998 due to this side effect[11].

### IL-3, -6, and -11

The interleukins IL-3, IL-6, and IL-11 produce a significant stimulation of platelet production. However, the clinical uses of recombinant IL-3 or IL-6 are severely

limited by their proinflammatory properties, which induce flu-like symptoms, including hypotension, fatigue, and myalgias[39]. However, recombinant human IL-11 (rhIL-11) was approved by the United States Food and Drug Administration for use in the prevention of chemotherapy-induced thrombocytopenia[40]. IL-11 acts synergistically with IL-3, TPO, and stem cell factors to increase the number and maturation of megakaryocytic progenitors[41]. IL-11 has also been shown to increase spleen megakaryocyte colony-forming cells and spleen colony-forming units in mice[41]. In patients with cancer who were receiving chemotherapy, a subcutaneous injection of rhIL-11 at a dose of 25-50 μg/kg per day increased their platelet levels and reduced their need for platelet transfusions[40,42]. In cirrhotic patients (Child A and B) with thrombocytopenia, a daily subcutaneous injection of rhIL-11 at 50 μg/kg per day improved platelet counts within 6 to 8 d from the treatment initiation, and the platelet counts doubled in 89% of the cirrhotic patients, achieving > 80 × $10^9$/L in 78%[43]. However, rhIL-11 can cause significant toxicity, including edema, fluid retention, cardiovascular events, and, in some studies, myalgias and arthralgias[44]. Therefore, the use of rhIL-11 in cirrhotic patients requires careful attention due to the high frequency of regimen-related toxicity[43].

### *Mimetics (TPO peptide and nonpeptide mimetics)*

Recently, great interest has been focused on the development of TPO peptide and nonpeptide mimetics. These mimetics are designed to bind to the TPO receptor but have no sequence homology with endogenous TPO. Two TPO receptor agonists (Romiplostim and Eltrombopag) are currently available.

Romiplostim is a 60 kDa molecule that is composed of 4 TPO-mimetic peptides that are attached *via* glycine bridges to an IgG heavy-chain Fc molecule[45]. This agent increased the platelet count in healthy individuals[46] and in patients with immune thrombocytopenia (ITP)[47-50] and myelodysplastic syndrome[51-53]. Romiplostim has been evaluated in patients with HCV-related thrombocytopenia. In patients with chronic liver disease and severe thrombocytopenia secondary to HCV infection, preoperative Romiplostim administration increased platelet counts to a level acceptable for elective surgical interventions without postoperative bleeding episodes[54]. Romiplostim is currently approved by the FDA only for the treatment of chronic ITP in adults and is administered weekly as a subcutaneous injection at a dose of 1 to 10 mg/kg.

Eltrombopag is a small-molecule nonpeptide oral platelet growth factor that acts as a TPO-R agonist. The binding of eltrombopag to the transmembrane domain of the TPO receptor activates intracellular signal transduction pathways that stimulate megakaryocyte proliferation and differentiation and increase platelet counts in a dose-dependent manner in healthy subjects and patients with chronic ITP[55,56]. Eltrombopag is FDA-approved for the treatment of adults with ITP and is administered orally at a dose of 25 to 75 mg per day (lower doses in patients of Asian ancestry). One potential advantage of Eltrombopag that makes it superior to the TPO peptide (Romiplostim) is that Eltrombopag may be administered orally. A phase Ⅱ, multicenter, randomized trial of daily Eltrombopag in patients with HCV-associated thrombocytopenia and compensated liver disease showed that, after 4 wk of therapy, platelet count increases to > 100 × $10^9$/L were achieved in 75%, 79%, and 95% of patients treated with 30 mg, 50 mg, and 75 mg Eltrombopag, respectively, compared to 0% of placebo patients ($P$ < 0.001)[57]. None of the patients with liver disease had a worsening of their liver function tests[57]. Additionally, significantly more patients in the Eltrombopag treatment groups completed 12 wk of antiviral therapy (36%, 53%, and 65% in the 30-mg, 50-mg, and 75-mg groups, respectively), compared with 6% of placebo patients[57]. The latest study revealed that Eltrombopag administration prior to elective invasive procedures reduced the need for platelet transfusions in patients with chronic liver disease compared with placebo, with platelet transfusion frequencies of 28% and 81%, respectively[58]. Nonetheless, the use of Eltrombopag has been reported to be associated with an increased incidence of portal-vein thrombosis compared to placebo, at 4% and 1%, respectively[58]. Collectively, although rhIL-11 and TPO mimetics are alternative tools for noninvasively treating thrombocytopenia due to liver cirrhosis, the evidence for their ability to improve thrombocytopenia is mainly based on patients with ITP; few studies have evaluated cirrhotic patients. A further investigation of their usefulness for the treatment of thrombocytopenia due to liver cirrhosis is expected through clinical trials.

## INTERVENTIONAL MANAGEMENT

### *PSE*

PSE is an interventional, non-surgical procedure that was developed to treat hypersplenism secondary to hepatic disease. PSE for hypersplenism can be carried out with almost no blood loss under local anesthesia. In 1973, Maddison[59] proposed total splenic embolization using interventional techniques instead of splenectomy for the treatment of hypersplenism. Initially, patients with complete splenic embolization had severe complications, including splenic abscess, splenic rupture, serious pneumonia, sepsis, hematoma, bleeding, pancreatic and hepatic infarction, and death. Thus, an important limitation of complete splenic infarction was the high incidence of morbidity. Six years later, Spigos *et al*[60] developed PSE and treated 13 patients with PSE, antibiotic coverage, and post-embolization pain control, which were performed safely and effectively. A significant reduction in both the morbidity and mortality rates was obtained by PSE compared with complete splenic embolization.

As for the clinical effects, PSE for hypersplenism has been reported to achieve prolonged improvement in blood cell counts[61,62]. The platelet count rises after PSE and then reaches the peak value in 1-2 wk. The platelet

counts stabilize in 2 mo at approximately 2-fold higher than their value before PSE, slightly less than the peak value. This PSE-derived clinical benefit is mainly due to the resolution of the platelet sequestration in the spleen. As an alternative possible mechanism of increasing the platelet counts after PSE, Hidaka *et al*[19] reported that TPO production, the score of megakaryocytes with platelet production, and an index of platelet production by megakaryocytes in the bone marrow were restored after PSE in cirrhotic patients but not in patients with idiopathic portal hypertension. In addition to the prolonged improvement in blood cell counts, several studies have reported PSE-associated fringe benefits, such as individual liver functional improvement[61,63]. The mechanism of these liver functional changes is not well elucidated but may be related to increases in hepatic arterial and superior mesenteric vasculature blood flow after embolization[64]. Porter *et al*[65] used a video dilution technique to demonstrate that the splenic arterial flow was reduced from 19% to 3% post-embolization, whereas there was a concomitant increase in the hepatic arterial flow from 3% to 15% and an increase in the superior mesenteric blood flow from 6% to 19%. However, Mukaiya *et al*[66] reported that PSE decreased splenic arterial flow and reduced splenic vein pressure without altering portal blood flow by a thermodilution method. Bárcena *et al*[67] reported that PSE could resolve splenic artery steal syndrome, which resulted in improved graft function in liver-transplanted patients.

PSE, like laparoscopic splenectomy, has recently been highlighted and widely applied to improve thrombocytopenia in cirrhotic patients prior to the administration of Peg-IFN and ribavirin for the treatment of HCV infections. PSE prior to IFN-based therapy, compared with no PSE, produced advantageous maintenance of higher platelet counts and an increase in adherence to Peg-IFN[23,68]. PSE can also be adapted to improve esophagogastric varices and portal hypertension in cirrhotic patients. In other medical treatments, such as hepatectomy for hepatocellular carcinoma[69], chemotherapy for cancer, and orthotopic liver transplantation[70,71], clinical benefits, such as improved thrombocytopenia and neutropenia, have been reported for PSE as a pre- and post-treatment procedure.

### *Splenic infarction ratio or splenic infarcted volume?*

Classically, the splenic infarction ratio ranges from 50% to 80%; this range has been used routinely as the target of PSE. While the splenic infarction rate correlated positively with increases in the platelet count, no therapeutic differences were found in patients with splenic infarction rates of 50%, 70% and 80%. Therefore, more reliable predictive factors of the increase in platelet counts after PSE and the recommended extent of splenic infarction in PSE for liver cirrhosis are needed. Recently, contrast-enhanced computed tomography (CT) scanning has enabled us to accurately measure the area of splenic infarction. A previous study by the present authors proposed the novel concept that infarcted splenic volume, and not the splenic infarction rate, is a determinant factor for increases in the platelet count after PSE and that an infarcted splenic volume of greater than 388 mL could induce a sufficient increase in the platelet count at 1 year after PSE [formula of the increased platelet counts at 1 year = 2.19 + 0.01 × infarcted splenic volume (mL), $R^2$ = 0.203][72]. This easy prediction method would help to determine the necessary infarcted splenic volume or spleen embolization ratio during PSE. As an adequate preoperative splenic volume and infarcted splenic volume are required for an effective increase in platelet counts after PSE, the PSE for cirrhotic patients with small spleens, such as those smaller than 400 mL, must embolize nearly the entire volume of the spleen (388 mL = 97% of 400 mL). Based on the above concept, which applies to cirrhotic patients with preoperative splenic volumes < 400 mL, a laparoscopic splenectomy, which can remove the total spleen, has been recommended to obtain as great an increase in the platelet counts as possible[62,72]. In cirrhotic patients with preoperative splenic volumes greater than 400 mL, a prolonged increase in the platelet counts could be achieved by PSE with sufficient infarcted splenic volume. As for the upper limit of infarcted splenic volume in a single PSE, however, there is evidence that a massive infarcted splenic volume of greater than 540 mL in a single PSE is a significant risk factor for severe complications, such as splenic abscess, refractory ascites, or pleural effusion post PSE[73]. Although recent advances in interventional radiology have further decreased the side-effects of PSE and have greatly expanded the indications of PSE, the morbidity rate of PSE for hypersplenism has still been shown to fall within the range of 0% to 17%[28,73-76]. As for mortality, some studies have reported that PSE for hypersplenism is associated with no deaths[28,76,77], whereas others have reported a mortality rate ranging from 1% to 12%[73-75,78].

A relapse in thrombocytopenia is occasionally observed in patients following PSE. In general, the residual splenic volume will decrease gradually over a period of months after PSE. In contrast, a re-enlargement of the residual splenic area after PSE, accompanied by a relapse of thrombocytopenia, has been reported in some cases[62]. Indeed, in cirrhotic patients with massive splenomegaly above 700 mL, the non-infarcted splenic volume plays an important role in long-term platelet increases[62]. In cases of relapse after PSE, laparoscopic splenectomy as a salvage treatment can provide a sufficient increase in platelet counts[79]. However, for an inexperienced surgeon, splenectomy following PSE is very difficult because of the inflammatory reaction around the spleen. As an alternative procedure, repeated PSE might be a safe and effective strategy against the relapse of thrombocytopenia post PSE. Furthermore, Child-Pugh class C is known as a significant risk factor of severe complications after PSE[73]. Additionally, in patients with Child-Pugh class C and massive splenomegaly above 1000 mL,

**Table 2 Approaches to treat thrombocytopenia induced by liver cirrhosis based on preoperative splenic volume[62]**

| Splenic volume | SV < 400 mL | 400 mL ≤ SV ≤ 700 mL | 700 mL < SV |
|---|---|---|---|
| Procedure | L-splenectomy | L-splenectomy<br>or<br>Single PSE | L-splenectomy<br>or<br>Repeated PSE |
| Target in PSE | | Infarcted splenic area<br>(infarcted splenic volume) | Non-infarcted splenic area<br>(infarcted splenic ratio and non-infarcted splenic volume) |

SV: Splenic volume; L-Splenectomy: Laparoscopic splenectomy; PSE: Partial splenic embolization. Modified and adapted from reference[62].

repeated PSE may be a safe and effective strategy to achieve a sufficient infarcted splenic volume and a smaller non-infarcted splenic volume. Therefore, depending on the preoperative splenic volume, laparoscopic splenectomy (preoperative splenic volume < 400 mL), a single PSE (400 mL < preoperative splenic volume < 700 mL), or repeated PSE (preoperative splenic volume > 700 mL) may be recommended[62]. This new approach, based on the preoperative splenic volume, will help us to select a suitable operative procedure for thrombocytopenia preoperatively, as shown in Table 2.

Thus, the volume of the embolized spleen has been found to be critical in PSE. If the embolization volume is too small, then the therapy will not be effective, and if it is too large, then the risk of serious complications is increased significantly. Therefore, the quantitative evaluation of the embolized volume during PSE is both desirable and useful. The blood flow rate has traditionally been used as an indicator of the embolized volume, but the results have largely depended on the experience of the operator. Making an accurate estimation of the embolized splenic volume during PSE is still difficult. Further developments in the technique of real time assessment for embolized splenic volume are expected to produce a safer and more effective PSE.

## SURGICAL MANAGEMENT

### *Splenectomy*

As a surgical option, open splenectomy has been performed for hypersplenism since the 1950s. Since the first report of laparoscopic splenectomy for ITP by Delaitre and Maignien[80] in 1991, recent advances in laparoscopic surgical techniques have enabled the performance of laparoscopic splenectomy, even for hypersplenism, with advantages over conditional open splenectomy that include less blood loss, less pain, a shorter hospital stay, better cosmetic outcomes, and fewer surgery-related complications[81-83]. Laparoscopic splenectomy can also be performed as a hand-assisted laparoscopic splenectomy (HALS) in cirrhotic patients with hypersplenism. Several studies have demonstrated that HALS is more appropriate than total laparoscopic splenectomy in cirrhotic patients with portal hypertension as laparoscopic splenectomy is technically difficult due to the splenomegaly, well-developed collateral circulation, and the increased risk of bleeding caused by thrombocytopenia. Ohta *et al*[84] reported that portal hypertension and severe liver dysfunction were independent risk factors for massive intraoperative bleeding during laparoscopic splenectomy. Furthermore, massive intraoperative hemorrhage, which is usually difficult to control by laparoscopic procedures alone, is a risk factor for serious postoperative morbidity in patients with liver dysfunction. The surgeon's use of one hand in HALS can control a sudden massive intraoperative hemorrhage and can easily mobilize a huge spleen, which is likely to result in less intraoperative blood loss and shorter operative times in comparison to conventional laparoscopic splenectomy.

Similar to PSE, laparoscopic splenectomy has been performed for hypersplenism-induced thrombocytopenia in patients with cirrhosis. Yoshida *et al*[85] reported that the platelet count after splenectomy in cirrhotic patients can be predicted based on preoperative clinical characteristics [the increased platelet count at 1 mo ($\times 10^9$/L) = 6.320 + 0.011 (preoperative splenic volume) - 0.004 (lymphocyte count/μL) + 2.25 (preoperative platelet count $\times 10^9$/L), $R^2 = 0.336$]. Thus, similar to PSE, the laparoscopic splenectomy-derived clinical benefit for thrombocytopenia depends on the preoperative splenic volume, indicating that preoperative splenic volume will help physicians to discern the primary cause of thrombocytopenia due to liver cirrhosis among splenic and other mechanisms, such as decreased TPO production (Figure 1).

The morbidity and mortality rates following laparoscopic splenectomy for hypersplenism are 11%-36% and 0%, respectively[86-88]. Among the severe complications that can occur after splenectomy, portal and splenic vein thrombosis (PSVT) and overwhelming post-splenectomy infection (OPSI) are well known adverse events. The former originates from hemodynamic changes that occur post-splenectomy, such as a complete lack of splenic vein flow following decreased portal vein flow. Recent advances in diagnostic modalities, such as enhanced CT or Doppler ultrasonography, reveal that PSVT is a common complication after splenectomy, despite the endogenous coagulopathy of cirrhosis[89-92]. Previous studies demonstrated that massive splenomegaly and the splenic vein diameter were significant risk factors for PSVT after laparoscopic splenectomy in cirrhotic patients[93,94]. Both the spleen size and splenic vein diameter are thought to be proportional to the splenic venous flow and pressure, suggesting that the rapid drop in the flow of the splenic vein after splenectomy contributes to thrombus formation. As the clinical manifestations of PSVT are

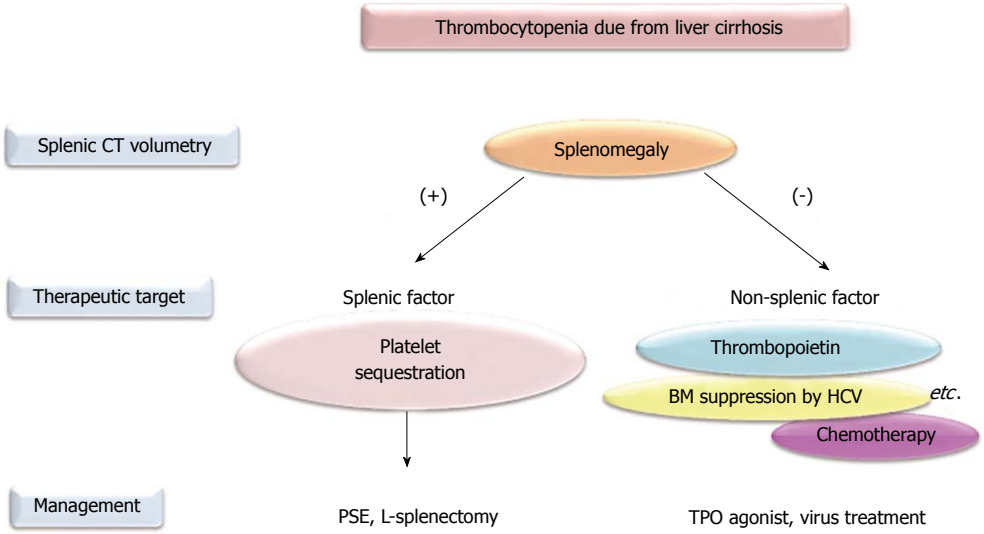


**Figure 1 Approaches to treat thrombocytopenia due to liver cirrhosis according to splenic volume.** Thrombocytopenia is defined as fewer than 100 × $10^9$/L. CT: Computed tomography; PSE: Partial splenic embolization; L-splenectomy: Laparoscopic splenectomy; BM: Bone marrow; HCV: Hepatitis C virus; TPO: Thrombopoietin.

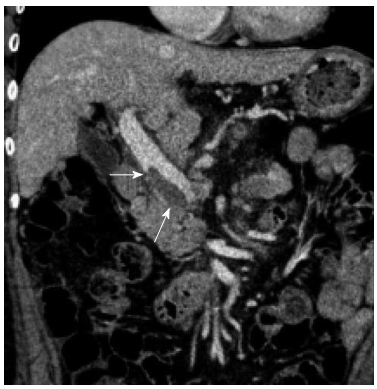

**Figure 2 Portal vein thrombosis after splenectomy (arrow).**

nonspecific, asymptomatic PSVT is diagnosed in 52.5% (21/40) of patients[94]. Among the types of PSVT, portal vein thrombosis (PVT) and mesenteric vein thrombus extending from the distal splenic vein should be treated with anti-coagulation therapy (Figure 2). Kawanaka *et al*[95] reported a 36% incidence of PVT after splenectomy in cirrhotic patients and a low antithrombin Ⅲ (AT-Ⅲ) activity; any further decreases in this activity were associated with PVT. They showed that treatment with AT-Ⅲ concentrates [prophylactic administration of AT-Ⅲ concentrates (1500 U/d)] on postoperative days 1, 2, and 3 was likely to prevent the development of PVT after laparoscopic splenectomy[95].

### *Overwhelming post-splenectomy infection*

The spleen contains many macrophages (part of the reticuloendothelial system), which are immune cells that phagocytose (eat) and destroy bacteria. Since overwhelming post-splenectomy infection (OPSI) was first described by King and Schumaker[96] in 1952, OPSI has become another well-known adverse event post-splenectomy. OPSI originates mainly from encapsulated bacteria, such as Streptococcus pneumoniae, Hemophilus influenzae, and Neisseria meningitidis. Because capsules made of polysaccharides (sugars) permit bacteria to evade phagocytosis by macrophages, opsonization is required for the complete phagocytosis of an encapsulated bacterium. The spleen produces immunoglobulin M antibodies and complements, both of which can be used to opsonize bacteria. Thus, the spleen plays an important role in both the tagging of bacteria for destruction and the actual destruction of the bacteria through phagocytosis. As infecting bacteria cannot be adequately opsonized in conditions such as post-splenectomy, this infection becomes more severe. OPSI is a rare but rapidly fatal infection that occurs in patients following the removal of the spleen. Therefore, post-splenectomy patients require immunizations (pneumococcal conjugate vaccine, Hib vaccine, and the meningococcal vaccine) against pathogens that normally require opsonization for phagocytosis by the splenic macrophages.

## CONCLUSION

The major mechanisms of thrombocytopenia in liver cirrhosis are (1) platelet sequestration in the spleen; and (2) decreased production of TPO in the liver. The concept of splenic volume helps us to discern the primary cause of thrombocytopenia due to liver cirrhosis among splenic sequestration and other mechanisms, such as decreased TPO production. For thrombocytopenia caused by platelet sequestration in the spleen, either PSE or laparoscopic splenectomy is effective against thrombocytopenia in cirrhotic patients. The choice between PSE and splenectomy depends upon the splenic volume, the intention of the treatment (required increase in platelet counts), and the conditions of the patient (whether gen-

**Table 3 Comparison between partial splenic embolization and laparoscopic splenectomy for thrombocytopenia caused by liver cirrhosis**

| Procedure | PSE | L-splenectomy (HALS) |
|---|---|---|
| Invasiveness | + | +++ |
| | (no transfusion) | (rarely with major bleeding) |
| | (local anesthesia) | (general anesthesia) |
| Platelet increase | ++ | +++ |
| Specific complication | Splenic abscess | Portal thrombosis, OPSI |
| Available in case with HCC | Synchronous TACE | Synchronous RFA or hepatectomy |

PSE: Partial splenic embolization; L-Splenectomy: Laparoscopic splenectomy; HALS: Hand-assisted laparoscopic splenectomy; OPSI: Overwhelming post-splenectomy infection; HCC: Hepatocellular carcinoma; TACE: Trans-arterial chemoembolization; RFA: Radio frequency ablation.

eral anesthesia is available) (Table 3). For thrombocytopenia caused by a decreased production of TPO, TPO agonists and targeted agents may represent alternative tools for noninvasive treatment of thrombocytopenia due to liver cirrhosis in the near future.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

# Autoantibodies in primary biliary cirrhosis: Recent progress in research on the pathogenetic and clinical significance

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## Abstract

Primary biliary cirrhosis (PBC) is a chronic progressive cholestatic liver disease characterized by immune-mediated destruction of the small- and medium-sized intrahepatic bile ducts and the presence of antimitochondrial antibodies (AMA) in the serum. AMA are detected in over 90% of patients with PBC, whereas their prevalence in the general population is extremely low, varying from 0.16% to 1%. Previous studies have shown that the unique characteristics of biliary epithelial cells undergoing apoptosis may result in a highly direct and very specific immune response to mitochondrial autoantigens. Moreover, recent studies have demonstrated that serum from AMA-positive PBC patients is reactive with a number of xenobiotic modified E2 subunits of the pyruvate dehydrogenase complex, which is not observed in the serum of normal individuals. These findings indicate that chemicals originating from the environment may be associated with a breakdown in the tolerance to mitochondrial autoantigens. While it is currently generally accepted that AMA are the most specific serological markers of PBC, more than 60 autoantibodies have been investigated in patients with PBC, and some have previously been considered specific to other autoimmune diseases. This review covers the recent progress in research on the pathogenetic and clinical significance of important autoantibodies in PBC. Determining the pathogenic role of those autoantibodies in PBC remains a priority of basic and clinical research.



**Key words:** Primary biliary cirrhosis; Autoantibodies; Anti-mitochondrial antibodies; Anticentromere antibodies; Anti-gp210 antibodies

**Core tip:** While the presence of antimitochondrial antibodies is pathognomonic to Primary biliary cirrhosis (PBC), more than 60 autoantibodies have been detected in patients with PBC. Antinuclear antibodies (ANA) become positive in approximately 30% to 50% of patients with PBC. Among ANA, anti-gp210 and anticentromere antibodies have been indicated as significant prognostic markers. Previous studies have shown that unique characteristics of biliary epithelial cells during apoptosis may result in the presence of a direct and specific immune response to mitochondrial autoantigens. Moreover, recent studies have indicated that chemicals originating from the environment are associated with a breakdown in the tolerance against mitochondrial autoantigens.

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## INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic progressive cholestatic liver disease characterized by immune-mediated destruction of small- and medium-sized intrahepatic bile ducts and the presence of antimitochondrial antibodies (AMA) in serum of affected patients[1,2]. PBC is considered a model autoimmune disease on the basis of several features, including the presence of a highly direct and very specific immune response to mitochondrial autoantigens, female predominance, and homogeneity among patients[3]. Despite the fact that the mitochondrial targets are ubiquitous proteins expressed in all nucleated cells, the immunopathology of PBC is characterized by the presence of $CD4^+$ and $CD8^+$ T-cell infiltrates in the liver and targeted destruction of biliary epithelial cells (BECs)[4,5]. This suggests that BECs may have unique immunological characteristics. While it is currently accepted that AMA are the most specific serological markers of PBC, more than 60 autoantibodies have been investigated in PBC patients, some having previously been considered specific for other autoimmune diseases[6].

The immune system has an extraordinary capacity of preventing self-antigens from provoking an inflammatory reaction. Therefore, the presence of autoantibodies is the consequence of a breakdown in or complete failure of B cell tolerance to corresponding autoantigens[7]. However, antibodies against self-antigens are also found in cancer, during massive tissue damage, and even in healthy subjects. Natural autoantibodies (NAAs) are immunoglobulins produced at tightly regulated levels in the complete absence of external antigenic stimulation[7]. Although NAAs have long been considered a sign of a breakdown in tolerance, these antibodies appear to play an important role in the innate immune system as a first line of defense against pathogens as well as in the prevention of autoimmune diseases[8,9]. It was recently demonstrated that NAAs bind to apoptotic cells and thereby facilitate their uptake by dendritic cells, thus preventing activation of the adaptive immune system by molecules released upon apoptosis, which may trigger autoimmune events[7,10]. Considering the many nonspecific autoantibodies are detected in patients with PBC, we speculate that these antibodies may be NAAs that are not associated with the development of autoimmunity. Interestingly, the majority of NAAs are of the IgM type, with a smaller proportion of IgG and IgA types[11]. The elevated serum IgM level, which is a well-known characteristic of PBC, may reflect such an increase of IgM-type NAAs, although recent studies have stressed that environmental factors also play a major role[12,13]. However, the importance of innate immunity, including NAAs, in autoimmune responses has been only recently appreciated, and its role remains to be clarified. The present review focuses mainly on recent progress in studies of the pathogenetic and clinical significance of the more significant autoantibodies detected in PBC patients.

## ANTIMITOCHONDRIAL ANTIBODIES

AMA are detected in over 90% of patients with PBC, whereas their prevalence in the general population is extremely low, varying between 0.16% and 1%, and only reaching 8% in hepatitis C virus (HCV)-infected patients[13]. AMA seropositivity is a strong predictor for the development of PBC. Mitchison *et al*[14] found that only two of 29 AMA-positive healthy patients had a normal liver histology pattern. Ten years later, 76% of those patients had developed clinical signs and symptoms of PBC[14,15]. Thus, despite their high predictive value, AMA are not useful for prognostication in terms of clinical course of PBC. Moreover, most studies indicate that AMA levels are unaffected by treatment[13].

The autoantigens of AMA have been identified as the E2 subunits of the 2-oxo-acid dehydrogenase complexes, including the E2 subunits of the pyruvate dehydrogenase complex (PDC-E2), branched chain 2-oxo acid dehydrogenase complex, 2-oxo-glutarate dehydrogenase complex, and the E3 binding protein of dihydrolipoamide dehydrogenase[5,16-18]. The AMA target antigens are all localized within the inner mitochondrial matrix and catalyze the oxidative decarboxylation of 2-oxo-acid acid substrates[5,19]. In approximately 95% of patients, AMA are directed towards the 74 kD mitochondrial polypeptide identified as PDC-E2. During apoptosis of BECs, PDC-E2 remains immunologically intact without being glutathiolated, and becomes the source of the PDC-E2 apotope. The term apotope specifies an epitope created during the processes of apoptosis[20,21]. PDC-E2 contained within apoptotic bodies can be recognized by circulating AMA, and the resulting apotope-AMA complex then stimulates the innate immune systems in genetically susceptible individuals[21]. Immune destruction is restricted to BECs due to their unique physiology and is exacerbated by retention of PDC-E2 in apoptotic blebs resulting from apoptosis of BECs[21]. Several previous studies of the liver in PBC patients have reported accentuated apical expression of PDC-E2 in BECs, and induction of PDC-E2 cell-surface expression during BEC autophagy[22]. The accumulation of autophagic vacuoles appears to be critical for the cell-surface expression of PDC-E2, and such expression may play a role in antigen presentation on BECs, followed by autoimmune-mediated cell injuries of BECs[22].

### *Association between xenobiotic and antimitochondrial antibodies*

Although bacteria and viruses may induce PBC through molecular mimicry, other environmental factors, xenobiotics, or chemical compounds foreign to a living organism may exert similar effects[18]. Previous analysis of the specificity of anti-PDC-E2 has revealed a number of anti-PDC-E2 antibody subpopulations that recognize either the PDC peptide, PDC peptide conjugated with lipoic acid, or lipoic acid itself[23,24]. The immunodominant

epitope of PDC-E2 is the lipoylated domain, and the lipoic acid residue attached to AMA epitopes is necessary for autoantibody binding[24]. Moreover, recent studies involving quantitative structure-activity relationship analysis have demonstrated that serum from AMA-positive PBC patients, but not that from controls, is reactive with a number of xenobiotic modified PDC-E2 structures[25]. These findings indicate that chemicals of environmental origin may be associated with the breakdown of tolerance to mitochondrial autoantigens.

## ANTINUCULEAR ANTIBODIES

Antinuclear antibodies (ANA) are serological markers that can be found in a wide variety of liver diseases (drug-induced, viral, alcoholic hepatitis, nonalcoholic steatohepatitis, AIH, PBC, and PSC) and non-hepatic autoimmune diseases (Hashimoto thyroiditis, systemic lupus erythematosus, Sjögren syndrome), as well as in a subgroup of healthy individuals, though usually at a low titer levels[12]. Antinuclear indirect immunofluorescence patterns are characterized by anti-multiple nuclear dots, rim-like/membrane antibodies and less specific anticentromere antibodies (ACA). The molecular target of anti-multiple nuclear dots is the protein sp100 and the promyelocytic leukemia (PML) protein[12,26]. The anti-rim-like/membranous antibodies are mainly represented by the protein glycoprotein (gp)-210[12,27]. Muratori *et al*[28] demonstrated that ANA were present in almost 50% of patients with PBC and that their prevalence reached 85% in AMA-negative sera. Specifically, 27% of patients had anti-sp100, 16% had anti-gp210, and 16% had anticentromere antibodies. In other reports, the prevalence of ANA in PBC patients has been approximately 30%-50%[12]. Although the significance and predictive value of ANA have been confirmed in other autoimmune diseases such as type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease, the predictive significance of ANA in PBC remains unclear[20,29].

### *Anticentromere antibodies*

In patients with calcinosis cutis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia (CREST) syndrome or limited cutaneous systemic sclerosis, ACA positivity may be as high as 50%-90%. Although they are not specific to systemic sclerosis, ACA are usually associated with a good prognosis for that condition[6,30]. In patients with PBC, the ACA positivity rate is approximately 30%[6]. Nakamura *et al*[31] conducted a retrospective multi-center cohort study of 276 patients with biopsy-confirmed PBC, and found that positivity for ACA was a significant risk factor for the development of portal hypertension. The findings from histological observations indicate that the presence of ACA is most significantly associated with a relatively severe ductular reaction[31]. In PBC, the production of cytokines or growth factors from inflammatory cells, rather than retention of bile constituents, is potentially critical for the induction of ductular reaction[32]. In chronic hepatitis, this type of ductular reaction is also known to promote fibrosis *via* the production of transforming growth factor-β, monocyte chemoattractant protein-1, and platelet-derived growth factor by proliferating ductular epithelia[33,34]. Therefore, it is reasonable to speculate that more severe ductular reaction may play a crucial role in the progression to portal hypertension in PBC patients who are ACA-positive. However, the mechanism of bile duct damage appears to lead to chronic cholestasis and development of biliary cirrhosis, even in ACA-positive patients[20,35].

### *Antinuclear pore antibodies (gp210)*

A number of nuclear antigens have been recognized as targets of ANA in patients with PBC, including several components of the nuclear pore complex, such as the gp210 and p62 proteins[6]. Gp210 is an integral glycoprotein of the nuclear pore consisting of three main domains: a large glycosylated luminal domain, a single hydrophobic transmembrane segment, and a short cytoplasmic tail. Gp210 is recognized by antibodies in approximately 25% of patient with PBC[6,36]. The association between the presence of anti-gp210 antibodies and the outcome of PBC was first reported by Itoh *et al*[37], and subsequently confirmed by several additional studies[38-40]. Ishibashi *et al*[20] and Nakamura *et al*[41] reported that PBC patients with consistently high levels of anti-gp210-C terminal peptide antibody have a higher risk of progression to end-stage hepatic failure than do those without such antibodies, or those in whom antibody levels are initially positive but drop to low levels after treatment with ursodeoxycholic acid. These reposts demonstrate that antibodies against nuclear pore complexes, especially gp210-C terminal peptides, may serve as important surrogate predictors of PBC progression to end-stage hepatic failure. It has been shown that the presence of anti-gp210 antibodies was most significantly associated with more severe interface hepatitis and lobular inflammation[31]. Furthermore, there was a tendency for relatively more severe ductopenia (ductular reaction) in late stages of disease anti-gp210-positive patients[31]. These findings suggest that two main processes - bile duct destruction and interface hepatitis - are more severe in PBC patients positive for anti-gp210 antibodies compared to those negative for anti-gp210 antibodies; thus, leading to more frequent progression to end-stage hepatic failure[20,31]. Nakamura *et al*[42] found that expression of gp210 antigens was increased on the nuclear envelope of epithelial cells in small bile ducts in the liver of PBC patients and that the intensity of gp210 staining by immunofluorescence was positively correlated with the intensity of inflammation around small bile ducts. These observations indicate that gp210 may be a target antigen, reactivity against which plays an important role in both bile duct destruction and interface hepatitis.

**Table 1 Serum autoantibodies in primary biliary cirrhosis**

| Autoantibody | Prevalence | Comments | Ref. |
|---|---|---|---|
| Related to the diagnosis and prognosis of PBC | | | |
| AMA | 90%-95% | Diagnostic value | [1] |
| Anti-sp100 | 30%-50% | Diagnostic value | [13,43-46] |
| Anti-gp210 | 30%-50% | Possible prognostic value (hepatic failure) | [6,31,36-42] |
| Anticentromere antibodies | 16%-30% | Possible prognostic value (portal hypertension) | [6,30-35] |
| Anti-p97/VCP | 12.5% | Possible prognostic value (favorable) | [48-50] |
| Anti-EPO | 52.5% | Diagnostic value Less peripheral eosinophils | [51] |
| Anti-β2GPI | 2%-15% | Possible prognostic Value (poor) | [52,53] |
| Related to the pathogenesis of PBC | | | |
| ASCA | 24.2% | Enhanced mucosal immunity | [54-56] |
| Anti-ClpP | 30%-47% | Infectious factor | [57,58] |
| Anti-β-subunit of bacterial RNA polymerase | 32.8% | Bacterial triggers | [59] |
| Related to other autoimmune diseases | | | |
| Anti-SMA | 8.0%-25.0% | PBC-AIH overlap | [6,40,60] |
| Anti-dsDNA | 17.0%-22.0% | PBC-AIH overlap | [39,61,62] |
| Anti-SSA | 5.0%-33.0% | Sjogren syndrome | [63,64] |
| Anti-CCP | 2.7%-4.0% | Rheumatoid Arthritis | [65-67] |

PBC: Primary biliary cirrhosis; CCP: Cyclic cirtullinated peptide; SMA: Smooth muscle antigen; ClpP: Caseinolytic protease P; ASCA: Anti-saccharomyces cerevisiae antibodies; EPO: Eosinophil peroxidase; β2GPI: β(2)-glycoprotein I; VCP: Valosin containing protein; AMA: Antimitochondrial antibodies.

### *Antinuclear dot antibodies (Anti-sp100)*

Sp100 was discovered in the context of leukemic transformation and as an autoantigen in PBC[43]. Sp100 antigen is a 480 amino acid peptide with a calculated molecular weight of 53 kDa that shows aberrant electrophoretic mobility to 100 kDa[44]. The prevalence of anti-sp100 antibodies in patients with PBC is approximately 25%. Anti-sp100 antibody, which has a specificity of 94%, has an important diagnostic role in PBC, particularly in AMA-negative patients[26,45,46]. However, anti-sp100 antibodies have been increasingly found in many other autoimmune diseases, including systemic lupus erythematosus and systemic sclerosis. Interestingly, among PBC patients, approximately 74% of those with urinary tract infections are positive for anti-sp100, whereas the positivity rate is only 4.8% in those without such infections[6,47]. Given the high specificity of anti-sp100 as an immunoserological hallmark of PBC, these findings support the hypothesis that some bacterial infections might be involved in the induction of PBC-specific autoimmunity.

## OTHER AUTOANTIBODIES IN PBC

Other autoantibodies against nuclear constituents (ds-DNA, ssDNA, histone, scl-70, Sm, SSA-SSB, RNP, Jo-1, U1RNP) have also been detected in PBC, mostly in conjunction with rheumatic co-morbidities[6]. Anti-p97/VCP (valosin containing protein) antibodies are detected in approximately 12.7% of PBC cases, and their presence suggests a less progressive disease course and a benign prognosis[48-50]. Eosinophil peroxidase (Anti-EPO) antibodies have been detected in 52.5% of patients with PBC and 29.0% of patients with AIH. PBC patients who were positive for anti-EPO antibodies had a significantly smaller number of peripheral eosinophils than did patients who were anti-EPO negative[51]. As mentioned above, more than 60 autoantibodies have been detected in PBC patients, but some are not specific for any disease, while some are thought to be more closely related to other autoimmune diseases[6]. Among those autoantibodies, the more significant autoantibodies detected in PBC patients are summarized in Table 1[6,13,48-68]. A comprehensive review of these autoantibodies in PBC was published by Hu *et al*[6] in 2010.

### *Line immune assay*

Previous studies of autoantibodies in PBC have primarily focused on only a single type of autoantibody, whereas the positivity pattern of different autoantibodies in a single serum sample, as well as their clinical significance, have not been elucidated. A line immune assay (LIA) kit that can simultaneously measure different autoantibodies known to be involved in autoimmune disease has recently become available and has been employed in clinical practice. Using a LIA kit that can detect 9 autoantibodies against AMA-M2, M2-3E (a fusion protein of the E2 subunits of alpha-2-oxoacid dehydrogenases of the inner mitochondrial membrane), sp100, PML, gp210, Ro-52, LKM-1 (liver-kidney microsomes-1), LC-1 (cytosolic liver antigen type 1), and SLA/LP (soluble liver antigen/liver-pancreas antigen), Saito *et al*[68] examined the prevalence and positivity pattern of those autoantibodies in 80 patients with PBC, 40 patients with AIH, and 16 patients with PBC-AIH overlap. They found that the prevalence of positivity for anti-sp100, anti-PML, anti-gp210, anti-Ro-52, and ACA were 13.8%, 8.7%, 40%, 27.5%, and 32.5% in PBC, respectively. In the PBC-AIH overlap group, the prevalence of both anti-gp210 (68.7%) and anti-Ro-52 (81.2%) were significantly higher than those in the PBC and AIH groups. The authors concluded that LIA is useful for the diagnosis of PBC and PBC-AIH overlap, although AMA-M2 should be measured by the conventional ELISA-based method, as LIA is less sensitive than ELISA in detecting AMA[68].

## CONCLUSION

We have provided an overview of the recent developments in the general understanding of the pathogenetic and clinical significance of the autoantibodies that significantly impact PBC. Although there have been substantial advances, determining the pathogenic role of autoantibodies in PBC remains a priority of basic and clinical research.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

# Non-invasive prediction of forthcoming cirrhosis-related complications

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## Abstract

In patients with chronic liver diseases, identification of significant liver fibrosis and cirrhosis is essential for determining treatment strategies, assessing therapeutic response, and stratifying long-term prognosis. Although liver biopsy remains the reference standard for evaluating the extent of liver fibrosis in patients with chronic liver diseases, several non-invasive methods have been developed as alternatives to liver biopsies. Some of these non-invasive methods have demonstrated clinical accuracy for diagnosing significant fibrosis or cirrhosis in many cross-sectional studies with the histological fibrosis stage as a reference standard. However, non-invasive methods cannot be fully validated through cross-sectional studies since liver biopsy is not a perfect surrogate endpoint marker. Accordingly, recent studies have focused on assessing the performance of non-invasive methods through long-term, longitudinal, follow-up studies with solid clinical endpoints related to advanced stages of liver fibrosis and cirrhosis. As a result, current view is that these alternative methods can independently predict future cirrhosis-related complications, such as hepatic decompensation, liver failure, hepatocellular carcinoma, or liver-related death. The clinical role of non-invasive models seems to be shifting from a simple tool for predicting the extent of fibrosis to a surveillance tool for predicting future liver-related events. In this article, we will summarize recent longitudinal studies of non-invasive methods for predicting forthcoming complications related to liver cirrhosis and discuss the clinical value of currently available non-invasive methods based on evidence from the literature.



**Key words:** Non-invasive model; Prediction; Cirrhosis; Complication; Liver-related events

**Core tip:** In this article, we summarized recent longitudinal studies of non-invasive methods - including transient elastography, European Liver Fibrosis scoring system, Fibrotest, and acoustic radiation force impulse technique - for predicting forthcoming complications related to liver cirrhosis. We also discussed the clinical value of currently available non-invasive methods based on evidence from the literature and finally proposed areas for future research directions.

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## INTRODUCTION

Viral hepatitis accounts for the majority of chronic liver diseases (CLDs) worldwide[1-4]. In the absence of appropriate therapeutic interventions, CLD can progress to significant liver fibrosis or cirrhosis, resulting in complications, such as portal hypertension, ascites, or hepatocellular carcinoma (HCC)[5-7]. In recent years, secondary prevention of CLD progression to significant liver fibrosis or cirrhosis has been quite successful due to the development and application of antiviral therapies. However, liver-related events (LREs), especially cirrhosis-related complications, occur despite appropriate management with antiviral treatments. These LREs are clinically important and abruptly change the prognosis of patients with CLD.

Therefore, the identification of significant liver fibrosis and cirrhosis is essential for determining treatment strategies, assessing therapeutic response, and stratifying long-term prognosis based on the risk of developing LREs. For evaluating the extent of liver fibrosis, liver biopsy remains the reference standard. However, liver biopsies are often considered "imperfect" surrogate markers for liver fibrosis because of inherent limitations, including invasiveness, risk of life-threatening complications, intra- and inter-observer variability, and sampling error[8-11]. Moreover, monitoring the extent of liver fibrosis through serial liver biopsies during antiviral or antifibrotic treatments is not feasible in practice.

Accordingly, several non-invasive methods have been developed as alternatives to liver biopsies for evaluating the extent of liver fibrosis in patients with CLDs. Alternative approaches include measuring serum hyaluronic acid levels, the ratio of aspartate aminotransferase (AST) to alanine aminotransferase (ALT), AST-to-platelet ratio index (APRI), age-spleen-platelet ratio index (ASPRI), Fibrosis-4 (FIB-4), Forns' index, Fibrotest (FT), FIBROSpect, Hepascore, Lok index, European Liver Fibrosis (ELF) panel scoring system, and transient elastography (TE, FibroScan®)[12-25]. The diagnostic performance of these non-invasive methods has been assessed in many cross-sectional studies by comparing the area-under-the-receiver operating curves (AUROCs) for these methods with the histological fibrosis stage as a reference standard. Accordingly, some of these tools have demonstrated clinical accuracy for diagnosing significant fibrosis or cirrhosis and have provided reproducible and reliable results[26-30].

However, liver biopsy is not a perfect surrogate endpoint marker for the aforementioned reasons. Thus, non-invasive methods cannot be fully validated through cross-sectional studies[31]. Recent studies have focused on assessing the performance of non-invasive methods through long-term, longitudinal, follow-up studies with solid clinical endpoints related to advanced stages of liver fibrosis and cirrhosis, including liver failure, hepatic decompensation, development of HCC, and/or liver-related death. These longitudinal studies do more than simply comparing the AUROCs with an imperfect histological reference. They also help to establish the clinical importance of non-invasive methods, including prediction of long-term prognosis[31,32]. Recent studies suggest that serial assessments of non-invasive methods reflect changes in the risk of developing LREs in patients with CLD[33]. Thus, non-invasive methods are proposed to be valuable tools for monitoring changes in prognosis, which cannot be assessed with a liver biopsy.

In this article, we will summarize recent longitudinal studies of non-invasive methods for predicting forthcoming complications related to liver cirrhosis, such as hepatic decompensation, liver failure, development of HCC, or liver-related death. We will also discuss the clinical value of currently available non-invasive methods based on evidence from the literature. Finally, we will discuss future research directions.

## TRANSIENT ELASTOGRAPHY

Measurement of liver stiffness (LS) by TE has been proposed as a new non-invasive method for assessing the degree of liver fibrosis. The ability of TE to predict development of cirrhosis-related complications has also been explored in several prospective, longitudinal studies (Table 1).

### *Gastroesophageal varices*

An important cirrhosis-related complication is the development of gastroesophageal varices and bleeding. Gastroesophageal varices are present in 30%-60% of patients with liver cirrhosis. Variceal bleeding is an independent predictor of mortality[34-36]. Although several cross-sectional studies[37-42] have reported significant correlations between TE and the presence of esophageal varices (EVs) and/or EV bleeding, the performance of TE alone as a predictor of EVs is far from satisfactory. Thus, it has been suggested that TE alone cannot replace esophagogastroduodenoscopy for identifying patients with EVs[43]. A novel prediction model was recently introduced by Kim *et al*[44]. This model combines TE with different pathophysiological parameters of liver cirrhosis. The LS-spleen diameter to platelet ratio score (LSPS) combines the LS measurement obtained by TE with the spleen diameter measured by ultrasonography and the platelet count from a routine complete blood count. The LSPS has excellent accuracy for diagnosing and predicting high-risk EVs in patients with compensated hepatitis B virus-related CLD (AUROC = 0.953; negative predictive value 94.7%, positive predictive value 93.3%).

Based on a follow-up prospective, longitudinal study, Kim *et al*[45] concluded that LSPS is a reliable method for predicting variceal bleeding in hepatitis B virus-related liver cirrhosis. In this study, patients with LSPS $\geqslant$ 5.5 showed higher cumulative incidences of EV bleeding during the follow-up period. Among those with high-risk EVs, those with an LSPS $\geqslant$ 6.5 were at an increased risk of developing variceal bleeding (Figure 1).

The clinical significance of LSPS for predicting EVs in patients with compensated cirrhosis of different eti-

**Table 1 Description of prognostic studies assessing non-invasive tools for the prediction of forthcoming cirrhosis-related complications**

| Ref. | Etiology of liver disease | *n* | Assessment modality[1] | Endpoint | AUROC |
|---|---|---|---|---|---|
| Kim *et al*[45] | LC (HBV) | 577 | **LSPS** | Esophageal variceal bleeding | 0.929 |
| Berzigotti *et al*[46] | LC (mostly HCV) | 117 (T)/ 56 (V) | **LSPS**, platelet count, spleen diameter, LS | Esophageal varices<br>Clinically significant portal hypertension | 0.882 (T), 0.808 (V)<br>0.918 (T), 0.906 (V) |
| Masuzaki *et al*[49] | CLD (HCV) | 866 | **LS** | Hepatocellular carcinoma | N/A |
| Jung *et al*[33] | LC (HBV) | 1130 | **LS** | Hepatocellular carcinoma | N/A |
| Kim *et al*[50] | LC (HBV) | 217 | **LS**, **LSPS** | Hepatic decompensation | 0.773 (LS); 0.790 (LSPS) |
| Kim *et al*[51] | CLD (HBV) | 128 | **LS** | Liver-related events | 0.772 |
| Chon *et al*[52] | CLD (HBV) | 1126 | **LS**, **LSPS**, ASPRI, P2/MS, FIB-4 | Hepatocellular carcinoma<br>Hepatic decompensation | 0.789 (LS); 0.790 (LSPS)<br>0.820 (LS); 0.848 (LSPS) |
| Kim *et al*[53] | CLD (HBV) on antiviral therapy | 162 | **LS** | Liver-related events | 0.736 |
| Kim *et al*[54] | LC (HBV) on antiviral therapy | 103 | **LS** | Liver-related events | N/A |
| [2]Singh *et al*[56] | CLD (various) | 7058 | **LS** | Liver-related events | N/A |
| Parkes *et al*[58] | CLD (various) | 457 | **ELF** | Liver-related events | 0.82 |
| Kim *et al*[62] | CLD (HBV) | 170 | **ELF** | Liver-related events | 0.808 |
| Vergniol *et al*[66] | CLD (HCV) | 1457 | **FT**, LS, APRI, FIB-4, LB | Overall survival (5-yr) | 0.861 (FT) |
| de Ledinghen *et al*[67] | CLD (HBV) | 600 | **FT**, LS, APRI, FIB-4, LB | Overall survival (5-yr) | 0.82 (FT) |
| Park *et al*[68] | CLD (HBV) | 151 | FT, LS, **FT + LS**, **FT × LS**, LB | Liver-related events | 0.748 (FT + LS); 0.868 (FT × LS) |
| [3]Morishita *et al*[77] | LC (HCV) | 135 | ARFI (**LS**) | Esophageal varices (high-risk) | 0.890 (0.868) |
| [3]Takuma *et al*[78] | LC (mostly HCV) | 340 | ARFI (**SS**, LS) | Esophageal varices (high-risk) | 0.993 (0.930) |

[1]Bold face denotes main assessment modality; [2]Meta-analysis; [3]Cross-sectional study. AUROC: Area-under-the-receiver-operating curve; LC: Liver cirrhosis; CLD: Chronic liver disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus; T: Training set; V: Validation set; LSPS: Liver stiffness-spleen diameter to platelet ratio score; LS: Liver stiffness; ASPRI: Age-spleen-platelet ratio index; ELF: European Liver Fibrosis; FT: Fibrotest; APRI: Aspartate aminotransferase to platelet ratio index; ARFI: Acoustic radiation force impulse; SS: Spleen stiffness; N/A: Not available.

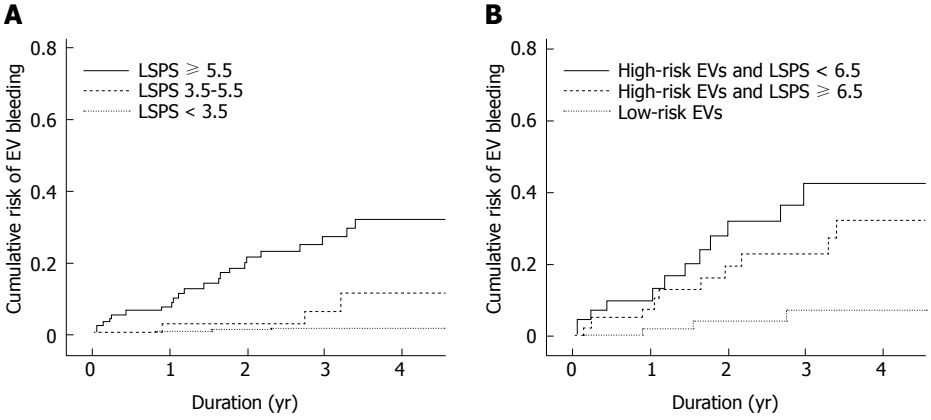



**Figure 1 Cumulative incidence rate of variceal bleeding based on liver stiffness measurement-spleen diameter to platelet ratio score values.** Reproduced with permission from Kim *et al*[45]. A: Among patients with liver cirrhosis related to hepatitis B virus, those with higher LSPS values had significantly higher cumulative incidences of bleeding from EVs during the follow-up period; *n* = 577; B: Among patients with high-risk EVs, patients with LSPS ⩾ 6.5 had higher risks for variceal bleeding; *n* = 150. LSPS: Liver stiffness measurement-spleen diameter to platelet ratio score; EV: Esophageal varice.

ologies was further confirmed by Berzigotti *et al*[46]. In this European multi-center study, which included a training set of 117 patients and a validation set of 56 patients with compensated cirrhosis, LSPS showed good diagnostic performance for predicting the presence of EVs (AUROC = 0.882; negative predictive value 90.8%, positive predictive values 73.2%). Patients with LSPS ⩾ 3.21 were considered to have EVs with an accuracy of 84.6% in the training set and 75.0% in the validation set. Interestingly, whereas the diagnostic performance of LSPS by AUROC was similar between the two studies[45,46], the cut-offs were different: 5.5 *vs* 3.21. The disparity between cut-offs is presumably due to differences in the etiologies of the CLDs. The original publication consisted of patients with hepatitis B virus-related liver cirrhosis only, whereas the latter study included patients with liver cirrhosis of various etiologies, mainly hepatitis C virus. The cross-sectional utility of LSPS demonstrated in Asian studies was validated in a European multicenter trial. However, similar to variceal bleeding, the utility of LSPS as a long-term prognostic factor should be validated through longitudinal, prospective studies performed by

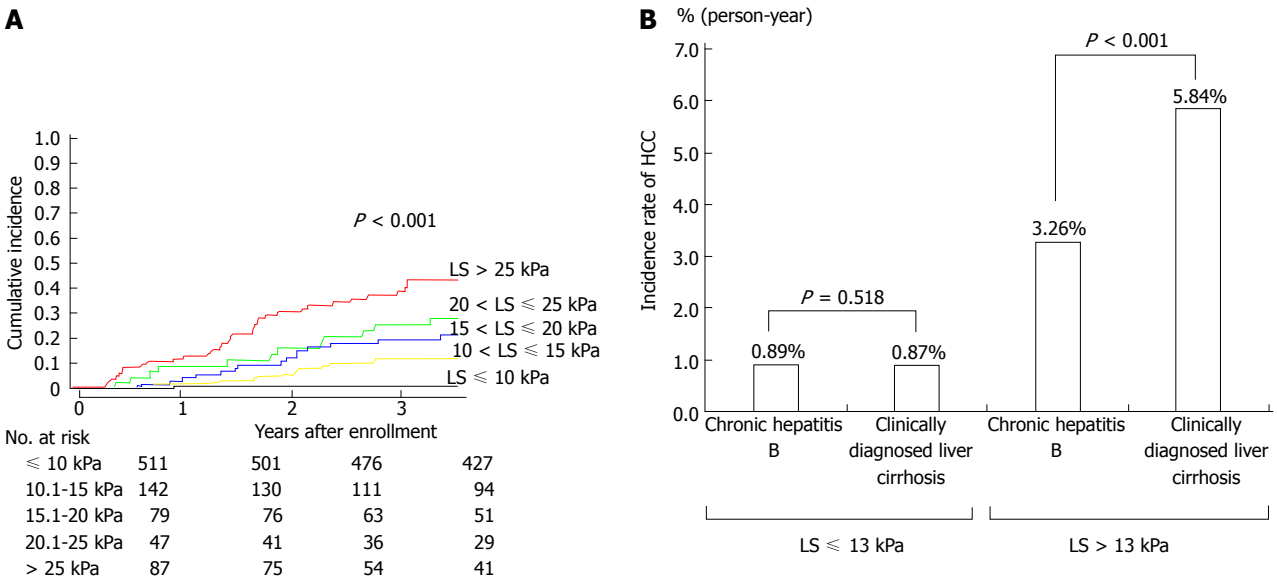



**Figure 2 Cumulative incidence of hepatocellular carcinoma.** A: The cumulative incidence of hepatocellular carcinoma (HCC) showed a step-wise increase according to the stratified liver stiffness (LS) measurement; *n* = 866. Reproduced with permission from Masuzaki *et al*[49]; B: Patients with higher LS values had significantly higher risks of developing HCC. Transient elastography (TE) and clinical criteria produced conflicting results for diagnosing liver cirrhosis. Patients diagnosed with liver cirrhosis based on TE findings were at higher risk of developing HCC than those diagnosed by clinical criteria (3.26% *vs* 0.87% per person-year); *n* = 1110. Reproduced with permission from Jung *et al*[33].

multiple investigators in patients of varying ethnicities and disease etiologies.

### *Hepatocellular carcinoma*

Advanced liver fibrosis and cirrhosis are regarded as major risk factors for the development of HCC. Early detection of HCC is critical for successful treatment[47,48]. In addition to the clinical application of TE for diagnosing the extent of liver fibrosis and predicting the development of gastroesophageal varices and hepatic decompression, recent studies suggest that TE can predict the development of HCC. Masuzaki *et al*[49] performed a large, prospective cohort study to assess the ability of TE to predict the development of HCC among Japanese patients with hepatitis C-related liver cirrhosis. In addition to age, male gender, and clinical cirrhosis, the LS value stratified by TE was identified as an independent risk factor for developing HCC. Compared to patients with LS values ≤ 10 kPa, patients with higher LS values had a significantly increased risk of developing HCC (LS values, 10.1-15 kPa, HR = 16.7; LS values, 15.1-20 kPa, HR = 20.9; LS values, 20.1-25 kPa, HR = 25.6; and LS values > 25 kPa, HR = 45.5). In addition, the cumulative incidence of HCC increased in a step-wise manner according to the stratified LS value (Figure 2A). These findings suggested that a greater extent of liver fibrosis, as assessed by higher LS values, was closely associated with an increased risk of developing HCC. Thus, TE is a clinically useful method for predicting development of HCC.

The clinical role of TE for predicting the development of HCC was further validated in patients with liver cirrhosis of different etiologies. Jung *et al*[33] conducted a study in Korean patients with chronic hepatitis B. In this large, prospective cohort study, a significant step-wise association was confirmed between LS values and the risk of developing HCC. Compared to patients with LS values ≤ 8 kPa, patients with higher LS values had significantly increased risks of developing HCC (LS values, 8.1-13 kPa, HR = 3.07; LS values, 13.1-18 kPa, HR = 4.68; LS values, 18.1-23 kPa, HR = 5.55; and LS values > 23 kPa, HR = 6.60). The diagnosis of liver cirrhosis may show conflicting results based on TE findings and clinical criteria. Patients diagnosed with liver cirrhosis based on TE findings showed increased risks of developing HCC than those diagnosed with liver cirrhosis based on clinical criteria (3.26% *vs* 0.87% per person-year) (Figure 2B). These findings indicate that TE might be a more precise indicator of compensated liver cirrhosis in comparison to clinical criteria. Furthermore, changes in the risk of developing HCC according to the pattern of changes in LS values were observed in the study. These data suggest that serial TE measurement may be a dynamic monitoring tool for estimating the risk of developing HCC in patients with liver cirrhosis.

These two large studies confirmed the clinical implications of TE as a non-invasive tool for assessing the risk of developing HCC. However, incorporation of TE into the routine surveillance strategy should be further investigated to determine if this increases the accuracy of detecting risk.

### *Hepatic decompensation and liver-related events*

Development of overall hepatic decompensation, not merely variceal bleeding, is another critical factor related to morbidity in advanced stages of liver fibrosis and cirrhosis. Hence, early prediction of hepatic decompensation is important for establishing therapeutic plans. Kim

*et al*[50] conducted a prospective, longitudinal study to assess the abilities of TE and LSPS to predict the first event of hepatic decompensation in patients with liver cirrhosis. The study included patients with cirrhosis related to hepatitis B virus without histories of hepatic decompensation, which was defined as a newly-developed ascites, hepatic encephalopathy, variceal bleeding, and/or deterioration of liver function to Child-Pugh class B or C. The risk of hepatic decompensation was stratified into three groups based on the LS or LSPS value. The results suggested that patients with higher LS values (13-18 and ≥ 18 kPa) had significantly higher risks of developing hepatic decompensation compared to those with lower values (< 13 kPa) (HR = 4.547, $P$ = 0.044 and HR = 12.446, $P$ < 0.001, respectively). Similarly, the risk of developing hepatic decompensation was increased in patients with higher LSPS values (1.1-2.5 and ≥ 2.5) compared to those with lower values (< 1.1) (HR = 5.796, $P$ = 0.004 and HR = 13.618, $P$ < 0.001, respectively).

Another study[51] investigated the potential role of TE for predicting the development of overall LREs in patients with hepatitis B virus-related CLD. Overall LREs included HCC, hepatic decompensation, and liver-related mortality. In this prospective study, 128 patients with chronic hepatitis B who were receiving antiviral therapy and had histologically-confirmed advanced liver fibrosis (≥ F3) and high viral loads (pre-treatment levels of HBV DNA ≥ 2000 IU/mL) were stratified into two groups based on the optimal cut-off (19 kPa). The patients with high LS values (≥ 19 kPa) had an increased risk of developing LREs compared with those who had lower LS values (< 19 kPa) (HR = 7.176; $P$ = 0.001). The incidence of LREs in patients with F3 and F4 were not statistically different (22.2% *vs* 13.6%; $P$ = 0.472). However, there was a significant difference in the incidence of LREs between patients with higher and lower LS values (44.4% in ≥ 19 kPa *vs* 6.9% in < 19 kPa; $P$ < 0.001). These results suggest that TE has superior performance for predicting LREs compared to that of histology for predicting long-term prognosis.

The role of TE-based non-invasive tools for predicting development of cirrhosis-related events was also analyzed in another study[52]. In this prospective study, the LS values and LSPS showed good performance for predicting the development of HCC (AUROC, 0.789 for LS value, 0.788 for LSPS) and hepatic decompensation (AUROC, 0.820 for LS value, 0.848 for LSPS). Furthermore, the LS value and LSPS were identified as independent predictors of HCC (HR = 1.040 for LS value, 1.001 for LSPS) and hepatic decompensation (HR = 1.033 for LS value, 1.002 for LSPS).

Antiviral treatments are actively administered to patients with hepatitis B virus. It is important to understand if TE can predict the long-term prognosis of patients with advanced liver fibrosis or cirrhosis who are being treated for hepatitis B viral infection. Recently, there were two publications attempting to answer this issue. One study[53] indicated that the LS value can predict the development of LREs in patients with chronic hepatitis B receiving entecavir therapy. Another prospective, longitudinal study[54] showed that stratified LS values based on the Laennec system for histologically subclassifying liver cirrhosis[55] and dynamic changes in LS values at follow up were significantly related to different risks of developing LREs in patients with advanced liver fibrosis who were receiving antiviral therapy for chronic hepatitis B virus infection.

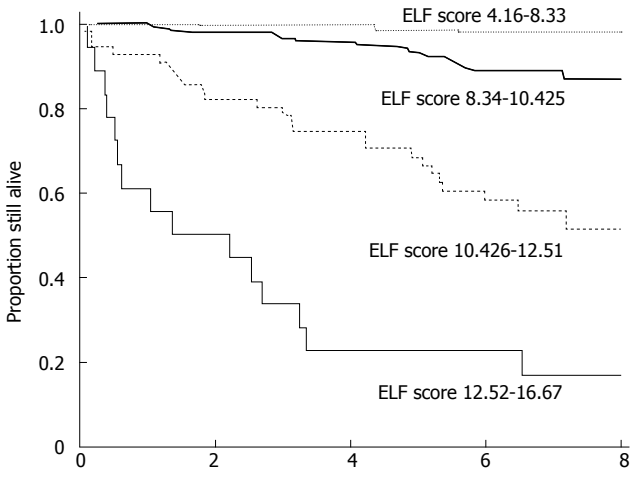
Recently, Singh *et al*[56] performed a comprehensive meta-analysis of 17 prospective cohort studies and confirmed the association between LS values and LREs in patients with CLDs. According to this meta-analysis, a greater degree of LS was significantly associated with an increased risk of developing LREs, including hepatic decompensation (RR = 1.07), HCC (RR = 1.11), overall mortality (RR = 1.22), or a composite of these events (RR = 1.32). Furthermore, a high LS value predicted the development of liver-related events, such as hepatic decompensation, HCC, and overall mortality, in a dose-dependent manner.

Thus, in addition to identifying liver fibrosis, TE appears to accurately predict long-term prognosis. However, several issues, such as determination of cut-off values, TE measurement interval, and cost-effectiveness, remain unresolved.

## ELF SCORING SYSTEM

Serum levels of extracellular matrix proteins and degradation products are increased in advanced liver fibrosis. The Original European Liver Fibrosis (OELF) panel of serum markers (aminoterminal propeptide of procollagen type Ⅲ, hyaluronic acid, and tissue inhibitor of matrix metalloproteinases-1), which reflects the dynamics of liver fibrosis, was combined with age in 2004[23]. It showed good diagnostic accuracy for evaluating liver fibrosis in CLDs of different etiologies[23]. Later, this panel was simplified to the ELF scoring system by removing age as a factor from the OELF panel, and the diagnostic accuracy remained intact[57]. ELF accurately predicted the extent of liver fibrosis in several cross-sectional validation studies[58-61]. However, there are limited studies on the longitudinal performance of the ELF scoring system for predicting LREs in patients with liver cirrhosis beyond the cross-sectional aspects of ELF.

Parkes *et al*[58] investigated the performance of the ELF scoring system for predicting the development of LREs in Caucasian patients with diverse etiologies of CLDs using paired ELF scores and liver biopsy data. In this multi-center study from the United Kingdom, the study population was stratified into four groups according to the ELF score for precise assessment of the risk of developing LREs. The performance of the ELF scoring system was compared with that of histopathology of a liver biopsy. The fully adjusted HRs for LREs showed a graded response according to the stratified ELF-score group. Compared to patients with ELF scores < 8.34,




| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | HR (95%CI) |
| ELF score 4.16-8.33 | 184 | 170 | 160 | 134 | 31 | 1 (reference) |
| ELF score 8.34-10.425 | 203 | 190 | 178 | 138 | 29 | 5 (1.4, 16.9) |
| ELF score 10.426-12.51 | 53 | 41 | 36 | 26 | 5 | 20 (5.5, 71.0) |
| ELF score 12.52-16.67 | 17 | 9 | 5 | 4 | 1 | 76 (17.6, 325.4) |

**Figure 3 Kaplan-Meier analysis of liver-related events in patients stratified according to enhanced liver fibrosis scores.** Reproduced with permission from Parkes *et al*[58]. The study population was stratified into four groups according to the enhanced liver fibrosis (ELF) score for precise assessment of the risks of developing liver-related events (LREs). LREs showed a graded response according to the stratified ELF score groups. Patients with higher ELF scores had significantly higher risks of having LREs; *n* = 457.

patients with higher ELF scores had significantly higher risks of developing LREs (ELF score, 12.52-16.67, HR = 75; ELF score, 10.426-12.51, HR = 20; and ELF score, 8.34-10.425, HR = 5) (Figure 3). Similarly, compared to patients with no fibrosis or mild fibrosis on liver biopsies, patients with advanced liver fibrosis were at increased risk of developing LREs (HR = 2.4 for moderate fibrosis; 8.3 for severe fibrosis/cirrhosis). Thus, the ELF scoring system was better than a liver biopsy for predicting development of LREs.

In addition, during the follow-up period, 82% of patients within the highest ELF-score group (12.52-16.67) experienced an LRE. In contrast, only 46% of those identified with severe fibrosis/cirrhosis based on a liver biopsy experienced an LRE. Moreover, when the risk of developing an LRE was analyzed with ELF as a continuous variable, the fully-adjusted OR for LRE was 2.2 (95%CI: 1.7-2.9). This result indicates that a unit increase in the ELF score was associated with a two-fold risk of developing an LRE. Furthermore, the ELF scoring system showed better diagnostic performance than a liver biopsy (AUROC, 0.82 for ELF, 0.70 for liver biopsy; $P = 0.004$) for predicting overall mortality.

Recently, the ELF scoring system was further confirmed to predict the development of LREs in a prospective study with a cohort of 170 Asian patients with hepatitis B virus-related CLDs[62]. The ELF scoring system showed good diagnostic performance for predicting the development of LREs (AUROC = 0.808). There was a significant association between the ELF score and the risk of developing LREs ($\rho = 0.449$; $P < 0.01$). A unit increase in the ELF score was associated with a 1.4-fold increase in the risk of developing an LRE. A higher ELF score at enrollment was correlated with an increased incidence of LREs (HR = 1.438; $P < 0.001$). Patients were subdivided into two groups based on a calculated optimal cut-off of 10.1. Patients with lower ELF scores had significantly reduced risks of developing LREs compared with those who had higher ELF scores (HR = 0.24; $P < 0.001$). Similar to the original publication[58], stratification of patients into three groups based on the ELF score showed that the fully-adjusted HRs for LREs had a graded response. Compared to patients with ELF score $\geqslant$ 10.40, patients with lower ELF scores had significantly lower risks of developing LREs (ELF score, < 8.10, HR = 0.072; and ELF score, 8.10-10.39, HR = 0.286). The superior diagnostic performance of the ELF scoring system for predicting overall mortality compared with a liver biopsy was also confirmed in this study (AUROC, 0.749 for ELF, 0.651 for liver biopsy).

Similar to TE, the ability of ELF to predict the development of cirrhosis-related complications has been confirmed in several studies. However, the clinical utility of the ELF scoring system is limited to centers at which extracellular matrix serum markers can be routinely measured. Thus, the general clinical applicability of the ELF scoring system requires extensive validation and a cost-effective analysis.

## FIBROTEST

The FT scoring system was developed by Imbert-Bismut *et al*[17] in 2001. The system is based on a panel of five serum markers, which includes α2-macroglobulin, haptoglobin, apolipoprotein A1, gamma-glutamyl transpeptidase (GGT), and total bilirubin adjusted by age and gender. The performance of FT as a surrogate marker for liver biopsy has been extensively studied. FT is ac-

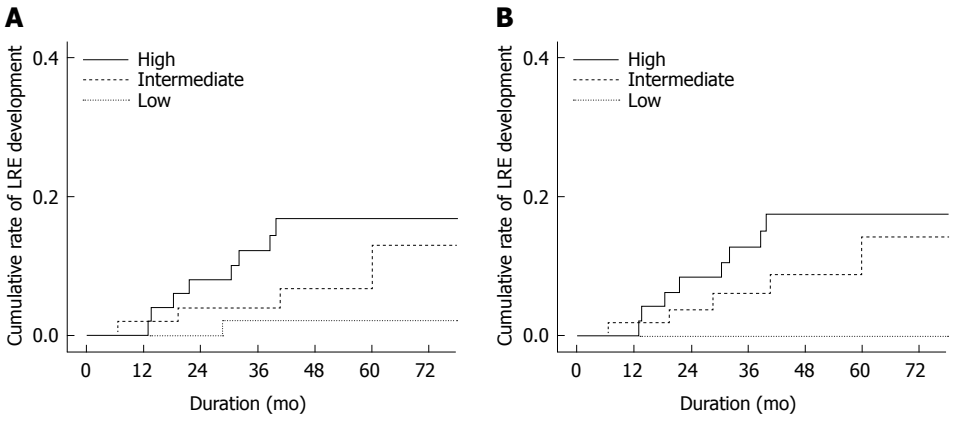



**Figure 4 Cumulative rate of liver-related events in patients stratified according to the combination of Fibrotest and liver stiffness values.** Reproduced with permission from Park *et al*[68]. The study population was stratified into three groups according to FT + LS (A) and FT × LS (B) values for precise assessment of the risk of developing LREs. The cumulative risk of developing LREs showed a significant graded response according to the stratified groups in both FT + LS ($P$ = 0.029) and FT × LS ($P$ = 0.008) analyses. $n$ = 151. FT: Fibrotest; LS: Liver stiffness; LRE: Liver-related event.

cepted as a promising non-invasive method with a high degree of accuracy and reproducibility for predicting advanced liver fibrosis and cirrhosis in patients with CLDs[63,64]. The prognostic value of FT for predicting five-year survival in patients with CLDs of various etiologies has been previously studied[65-67]. Vergniol *et al*[66] conducted a prospective five-year study to assess whether FT can predict overall survival (OS) in patients with chronic hepatitis C. In this study, FT showed a high level of accuracy for predicting OS with an AUROC of 0.861. The risk of mortality was increased with a cut-off value of 7.4 (adjusted HR = 90). Furthermore, risk stratification based on the FT score was able to predict five-year OS (FT, ≤ 0.75, OS = 97%; FT, 0.75-0.80, OS = 87%; FT, 0.81-0.85, OS = 84%; FT, 0.86-0.90, OS = 75%; FT, 0.91-0.95, OS = 69%; and FT > 0.95, OS = 0%). Comparable results were observed in a similar prospective, single-center study in patients with chronic hepatitis B[67]. The prognostic performance of FT for predicting OS was good with an AUROC of 0.82. The five-year OS rates were 96.8% for FT ≤ 0.73 and 49.2% for FT > 0.85, respectively.

The FT and measurement of LS by TE have high degrees of accuracy and reproducibility for predicting the extent of liver fibrosis and development of LREs in patients with CLDs. Thus, combined application of these two methods may provide additional discriminatory potential for predicting LREs. Indeed, the combination of LS and FT increased the cross-sectional diagnostic performance for assessing the degree of liver fibrosis and the diagnosis of liver cirrhosis (AUROC, 0.910 for LS only; 0.866 for FT only; 0.915 for FT + LS; and 0.929 for FT × LS)[64]. Park *et al*[68] recently conducted a prospective five-year longitudinal study to assess if the combination of FT with LS values enhanced the overall performance of predicting LREs in patients with hepatitis B virus-related liver cirrhosis. The combination was calculated as either the sum or multiplication of the FT score and LS values (FT + LS or FT × LS, respectively). In this study, 151 patients were analyzed, and the time-dependent AUROCs for predicting LREs were 0.748 (95%CI: 0.621-0.868) and 0.785 (95%CI: 0.647-0.906) at five-year follow-ups of FT + LS and FT × LS, respectively. Data were adjusted for age and histological fibrosis staging, which were the two significant factors according to univariate analysis ($P$ < 0.05). Multivariate analysis revealed that FT + LS and FT × LS were independent predictors of LREs ($P$ = 0.001, HR = 1.116; $P$ = 0.001, HR = 1.137, respectively). Stratification of the study population into three groups showed that the cumulative risk of developing LREs was significantly graded according to the stratification. The study population was stratified with cut-off values of 9.4 and 17.5 for the FT + LS analysis. The cumulative incidence rates of developing LREs at 2, 3, and 5 years were 2.1%, 4.4%, and 6.7%, respectively, for the low group (FT + LS < 9.4); 3.9%, 6.7%, and 13.0%, respectively, for the intermediate group (FT + LS 9.4-17.5); and 8.0%, 12.2%, and 16.8%, respectively, for the high group (FT + LS > 17.5) ($P$ = 0.029). Similarly, for the FT × LS analysis, the study population was stratified with cut-off values of 3.6 and 11.0. The cumulative incidence rates of developing LREs at 2, 3, and 5 years were 1.8%, 2.7%, and 4.5%, respectively, for the low group (FT × LS < 3.6); 3.8%, 6.1%, and 14.2%, respectively, for the intermediate group (FT × LS 3.6-11.0); and 8.3%, 12.8%, and 17.5%, respectively, for the high group (FT × LS > 11.0) ($P$ = 0.008).

The prognostic role of FT was demonstrated exclusively in a French study of a cohort of patients with chronic hepatitis C. Thus, the clinical value of FT requires further validation in a diverse population of patients with CLDs of varying etiologies. Fortunately, the prognostic performance of FT has recently been demonstrated in several Asian studies of a cohort of patients with chronic hepatitis B in cross-sectional and longitudinal studies[64,69]. Based on current findings, further research is warranted to define the clinical utility of FT for predicting the long-term prognosis of patients with liver cirrhosis.

## OTHER MODELS

Several simple non-invasive methods including APRI, FIB-4, Forns' index and Lok index have been found to be useful in assessing the extent of fibrosis in patients with CLD. Given their universal availability without additional economic burden, several studies evaluated the clinical significance of these simple non-invasive scoring systems for prediction of forthcoming complications related to liver cirrhosis.

A retrospective, multicenter, large-scale study of cirrhotic patients by Sebastiani *et al*[70] demonstrated that a combination of the Lok index and the Forns' index had a high negative predictive value (> 90%) to exclude clinically relevant EVs with an AUROC of 0.80, and suggested that these non-invasive markers may be useful as an initial screening tool for cirrhotic patients to exclude the presence of clinically relevant EVs. Another study by Angulo *et al*[71] showed that simple non-invasive methods including APRI and FIB-4 can identify patients with nonalcoholic fatty liver disease at higher risk for development of LREs and higher overall mortality. However, due to the limitations inherent in the retrospective studies, the clinical value of these non-invasive methods needs to be further validated in additional large-scale, prospective, and preferably, longitudinal studies.

Acoustic radiation force impulse (ARFI) elastography is a novel technology based on conventional B-mode ultrasonography, which provides numeric measurements of tissue stiffness as the shear wave velocity[72]. Several publications suggest that LS values and spleen stiffness measured by ARFI elastography both correlate well with the extent of liver fibrosis in patients with CLDs[73-75]. The role of ARFI elastography for assessing the risk of cirrhosis-related complications has been investigated in several studies[76-79].

A recent cross-sectional study by Morishita *et al*[77] showed that LS measurements by ARFI elastography predicted the presence of EVs (cut-off value of 2.05 m/s) or high-risk EVs (cut-off value of 2.39 m/s) in patients with hepatitis C virus-related liver cirrhosis. The diagnostic performance of this method was good (AUROC, 0.890 for EVs; 0.868 for high-risk EVs) with a high negative-predictive value (81% for EVs; 89% for high-risk EVs) and an acceptable positive-predictive value (78% for EVs; 69% for high-risk EVs).

In addition, a study by Bota *et al*[79] proposed a scoring system for predicting high-risk EVs based on liver stiffness and spleen stiffness assessed by ARFI elastography. The diagnostic performance of this scoring system was acceptable (AUROC = 0.721), yet further validation is desired.

Several studies[78,80] also reported that spleen stiffness measurement by TE can provide non-invasive assessment of the presence of EVs in patients with liver cirrhosis. Recently, a prospective study by Takuma *et al*[78] showed that spleen stiffness assessed by ARFI elastography was able to predict the presence of EVs (cut-off value of 3.18 m/s) or high-risk EVs (cut-off value of 3.30 m/s) in patients with liver cirrhosis. This method showed excellent diagnostic performance (AUROC, 0.933 for EVs; 0.930 for high-risk EVs) and a high negative-predictive value (98.4% for EVs; 99.4% for high-risk EVs). However, the positive-predictive value was low (61.0% for EVs; 47.8% for high-risk EVs). In contrast, Vermehren *et al*[81] reported that the diagnostic performance of spleen stiffness measured by ARFI elastography for predicting large EVs was not satisfactory (AUROC = 0.58). Moreover, Mori *et al*[82] showed that there was no significant association between spleen stiffness measured by ARFI elastography and the presence of EVs in patients with chronic hepatitis C. Furthermore, a recent meta-analysis conducted by Singh *et al*[83] showed that the current techniques of spleen stiffness measurement - either by ARFI or TE - are suboptimal for predicting EVs in patients with CLDs. Hence, further well-designed prospective studies are desired for evaluating the diagnostic role of spleen stiffness measurement in predicting EVs.

There have been no longitudinal studies of the role of LS measurements by ARFI elastography and spleen stiffness measurements with TE or ARFI elastography for risk assessment of LREs in patients with CLDs. Therefore, longitudinal, multi-center studies are warranted to establish a standardized protocol for measuring LS values by ARFI elastography and spleen stiffness by TE or ARFI elastography. Further studies are also needed to validate the clinical role of these non-invasive tools for assessing the risk of liver cirrhosis-related complications.

## CONCLUSION

The current view from a limited number of studies is that the recently-introduced non-invasive models can independently predict future cirrhosis-related complications, such as hepatic decompensation, liver failure, HCC, or liver-related death. In addition, some non-invasive tools, such as TE or TE-based models (*i.e.*, LSPS), appear to exhibit superior performance compared to histology and other non-invasive methods for predicting development of LREs.

Non-invasive models may no longer simply be tools for predicting the extent of fibrosis in patients with CLDs. Instead, current literature supports the use of these methods as surveillance tools for predicting future LREs and determining long-term prognosis, which will help to make informed treatment decisions. Future studies should assess whether serial changes in non-invasive measurements or scores reflect the dynamic changes in the risk of developing LREs. In addition, the combination of two or more non-invasive methods should be tested to determine if this approach provides greater discriminatory power for predicting LREs. Significant progress in this field will allow new strategies for risk management in patients with CLD to be developed in the near future.

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REVIEW

# Hepatitis C virus, mitochondria and auto/mitophagy: Exploiting a host defense mechanism

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## Abstract

Hepatitis C virus (HCV) is the major reason for liver transplantation and the main cause of liver-related morbidity and mortality in a great number of countries. As for the other viruses, this pathogen interferes in more than one process and in more than one way with host cell biology. A mounting body of evidence points, in particular, toward the drastic alterations of mitochondrial physiology and functions that virus is able to induce, albeit the mechanisms have partly remained elusive. Role of the mitochondria in immunity and in quality control systems, as autophagy, as well as the strategies that HCV has evolved to evade and even to manipulate mitochondrial surveillance for its benefit, highlights the importance of deepening the mechanisms that modulate this virus-mitochondrion interaction, not only to intensify our knowledge of the HCV infection pathogenesis but also to design efficient antiviral strategies.



**Key words:** Hepatitis C virus; Mitochondrial dysfunctions; Immunity; Autophagy; Mitophagy

**Core tip:** Among the strategies that host cells have evolved in the hard fight for their survival against viruses, auto/mitophagy processes have an emerging role. As preferential targets of hepatitis C virus (HCV) attack, mitochondria effectively establish themselves as an integral part of the host cell defense and mitophagy, as very recently unveiled, seriously impacts the course of hepatitis C infection. Aim of this review is to explore the current literature about the mechanisms that regulate the critical interplay between HCV and mitochondria, with particular regard to the strategies that the virus evolved to subvert and manipulate the auto/mitophagy pathways to its purposes.

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## INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide. It is a small-enveloped positive-strand RNA virus whose natural targets are hepatocytes and, possibly, B lymphocytes[1,2]. HCV belongs to the Hepacivirus genus of the family of Flaviviridae, that also contains the classical flaviviruses such as dengue virus and yellow fever virus, and chronically infects 120 to 180 million people worldwide[3,4]. The mechanisms of liver injury in chronic HCV infection are poorly understood though an emerging feature in the pathogenesis of the HCV-related disease is the presence of dysfunctions of mitochondria,

proved to be targets of viral proteins. A novel pathogenetic aspect about HCV-mitochondria interaction has recently emerged, disclosing HCV aptitude to hijack mitophagy pathway, a pivotal mitochondrial quality control system.

## HCV: FROM MOLECULAR VIROLOGY TO PATHOGENESIS OF INFECTION

HCV genome, an uncapped RNA molecule of approximately 9.6 kb, is composed of an open reading frame encoding a polyprotein precursor of approximately 3000 amino acids, flanked at the 5' and 3' ends by non-translated regions (NTRs) that are highly conserved among different HCV isolates and contain essential structural motifs exerting critical regulatory functions in viral RNA translation and replication processes[5].

HCV entry into the host cell is mediated by complex processes including the timely coordinated interaction of virus particles with cell surface receptors and entry factors like the low density lipoprotein receptor, the tetraspanin CD81, and the tight junction proteins claudin 1 and occludin[1], the HCV clathrin-mediated endocytic uptake of the lipoviral particle within an acidic endosomal compartment, followed by the pH-dependent fusion of the viral and endosomal membranes, essential for the release of genome-containing viral nucleocapsid into the cytosol[6]. The positive-strand genomic RNA, free in the cell cytoplasm following decapsidation of viral nucleocapsids, serves together with newly synthesized RNA, as messenger RNA for synthesis of the HCV polyprotein precursor. HCV genome translation is under the control of an internal ribosome entry site within the 5' NTR and produces a large polyprotein that undergoes proteolytic cleavage catalysed by host and viral proteases[7].

The co- and post-translational processing of protein precursor leads to the mature structural core protein and the E1 and E2 envelope glycoproteins, as well as to the nonstructural proteins p7 viroporin, NS2, the NS3 serine protease, the NS4A, NS4B, NS5A proteins and the NS5B RNA-dependent RNA polymerase[3,5] involved in the enzymatic functions of viral replication and processing of the viral polyprotein.

The core protein is a RNA-binding protein involved in the viral nucleocapsid assembly. This highly basic membrane protein contains two domains and is able to interact with a growing list of cellular proteins and to regulate a variety of host cell biological processes such as apoptosis, cell trasformation and immune modulation[8]. The envelope glycoproteins E1 and E2 are type I transmembrane proteins with N-terminal ectodomains and short C-terminal transmembrane domains that heterodimerize to form a non-covalent complex, which presumably represents the building block for the viral envelope[9]. However, the structure of the HCV E1-E2 complex, as well as the processes that are involved in HCV attachment, entry and fusion remain virtually unknown[5]. It is known, instead, that p7 is a small ion channel protein, composed of two transmembrane domains, that is crucial for production of infectious viral progeny but not for RNA replication[10]. Another non-structural protein dispensable for RNA replication is NS2, the viral autoprotease essential for the cleavage of the polyprotein precursor at the NS2-NS3 junction, that is crucial for the viral assembly. NS3 is a multifunctional protein harbouring a N-terminal HCV serine protease, which mediates the downstream cleavage events in the nonstructural region and a C-terminal RNA helicase-NTPase, which is vital for HCV RNA replication[3]. NS3 forms a stable complex with its cofactor NS4[11], that plays a major role in regulating NS3 helicase activity and a variety of NS3-mediated processes[12]. NS4B is the least characterized HCV protein and its role is not well understood even if it is known that its expression triggers a specific membrane alteration, designated as "membranous web", probably forming the scaffolds necessary for virus multiplication[5]. NS5A is a membrane-associated phosphoprotein whose functions are strongly debated, although it has been demonstrated that its modifications affect HCV replication and that it is potentially involved in HCV response to interferon[11,13,14]. Finally, the NS5B RNA dependent RNA polymerase (RdRp) is a fundamental enzyme in the promotion of synthesis of new RNA genomes and it is considered a major target for anti-HCV intervention[1,4].

As for all positive-strand RNA viruses, HCV RNA replication occurs on modified intracellular membranes, forming the above-mentioned endoplasmic reticulum (ER)-derived membranous web, in a poorly understood process. Remodeled intracellular membranes serve as a scaffold for the assembly of the replication complex composed of viral proteins, cellular components, and nascent RNA strands[15]. HCV replication is thought to be a semiconservative and asymmetric process, catalyzed by the NS5B RdRp, in which the positive strand RNA genome serves as a template for the NS5B RdRp to generate the negative strand replicative intermediate, that will be used, subsequently, as a template to produce further positive sense genomes. Finally, the numerous strands of positive polarity produced can be translated to produce new viral proteins, or used for further RNA replication, or be packaged into new virus particles[16].

The HCV particles are composed of HCV RNA genome, core and the envelope glycoproteins, E1 and E2, and are characterized by an irregular and globular shape with a mean diameter of 100 nm. Typically, they also show a tight association with cellular lipoproteins and lipids that affect both morphology and biophysical properties of the virion[17]. Although the molecular events that regulate the assembly and the release of infectious HCV particles have yet to be understood, the interaction of viral core protein assembly with intracellular lipid droplet structures is presumable. In addition to viral factors, several host cell factors have been described as participating in HCV assembly, including components of the very-low-density lipoprotein synthesis and secretion pathway, like the apolipoprotein E[18]. Once a newly

produced HCV nucleocapsid is formed in the cytoplasm and it acquires an envelope by budding through an intracellular membrane, virus particle is released from the cell through the constitutive secretory pathway[16].

More than 10 trillion virion particles are produced per day, even in the chronic phase of infection and, as a small RNA virus with an error-prone RNA polymerase, HCV exhibits enormous genetic variability, strongly challenging the host immune-mediated control. Six distinct genotypes and multiple subtypes have so far been identified, associated to specific clinical aspects including response to antiviral treatment. Despite international research efforts, much remains to be defined regarding clinical course of HCV infection and pathogenesis, mainly due to the frequent silent onset of the acute phase, as well as the asymptomatic early stages of chronic infection. Moreover, although chronic liver diseases such as hepatitis, cirrhosis and hepatocellular carcinoma, are considered the archetypal HCV-associated diseases, HCV is also involved in the pathogenesis of a number of extrahepatic manifestations like autoimmune and rheumatic disorders as well B-cell lymphoproliferative diseases[19,20]. However, morbidity and mortality of persistent HCV infection are mainly due to hepatic fibrosis and to the subsequent occurrence of cirrhosis and hepatocellular carcinoma[21]. Both innate and adaptive immune responses are the first barrier against HCV infection and multiple and complex HCV interactions with the host immune system, including elusion mechanisms, have been described. As well as being a crucial line of defense in the fight against the virus, immune response, mainly based on T lymphocytes and helper T cells intervention, represents, on the other side, the main cause of liver collateral damage in a long-lasting inflammation and fibrosis context. A qualitatively insufficient $CD8^+$ T lymphocyte responses, as well as viral inhibition of host defense strategies and the presence of multiple viral species in the same patient are probable reasons for the HCV persistence[20,22]. Therefore, immunotherapeutic strategies designed to reinforce the cellular immune response against HCV are largely attractive, even if any step of the HCV life cycle is a potential novel target of antiviral therapy[16]. Several antiviral agents directly targeting HCV life cycle, including NS3/4A protease, NS5B and NS5A inhibitors, have been clinically approved whereas promising host-targeting antiviral agents, offering the benefit of virtually pangenotypic activity, like inhibitors of cyclophilin A and of miR122 have advanced to phase 2 or 3 clinical trials. Extraordinary progress in the molecular virology of HCV has been done so far, but a further effort in the understanding of the viral life cycle and pathogenesis should improve therapeutic and preventive strategies for HCV infection[23,24].

## HCV AND MITOCHONDRIA: A LIFE AND DEATH STRUGGLE

As clinically observed and widely experimentally confirmed, mitochondria are one of the favorite targets of HCV attack. As sensors of the cell energy status, these organelles are involved in a myriad of cell physiological functions and are essential for crucial decisions like cell death, growth, proliferation and differentiation[25]. It is not surprising, therefore, that the HCV impact on mitochondria deeply affects different molecular pathways. Liver samples from patients with chronic hepatitis C typically show mitochondrial dysfunction along with ultrastructural abnormalities and oxidative stress associated to the presence of lipid peroxide-protein adducts and glutathione depletion, as well[26-28]. A correlation between the presence of oxidative protein derivatives and disease severity has also been shown and, concurrently, a decrease of oxidative stress markers has been found to be associated with viral eradication[29,30]. Chronic hepatitis C patients' oxidative stress phenotype has been successfully reproduced in transgenic mouse models for the hepatic disease with an increased lipid peroxidation and an oxidized mitochondrial glutathione pool reported[31]. Multiple and complex mechanisms of HCV interference with mitochondrial functions have been described and, although the consequences of this interplay remain to be elucidated, it has a considerable impact on the pathogenesis of infection, contributing to HCV evasion of the host innate immune response and infection persistence and, at the same time, potentially favoring both fibrogenesis and carcinogenesis processes[32]. The molecular mechanisms underlying the HCV-mitochondria interplay and how this may affect the viral lifecycle and translate in pathogenic effects for the host are not clear issues yet. As supported by increasing experimental data, HCV core protein is mainly responsible for the viral damaging action on mitochondria. Different phenomena, principally the widely observed increased mitochondrial reactive oxygen species (ROS) production seem to be, in fact, a direct consequence of core protein interactions with mitochondria, irrespectively of the context, either in infection, full-length replicon-bearing cells or in over-expression systems[33]. Even though core protein represents the paradigm of HCV-induced mitochondria impairment, other viral proteins have been shown to directly interact with mitochondria into the matrix or intermembrane space, or by binding to the outer membrane or to membrane sites closely associated with the ER (the mitochondria associated membranes, MAMs), supporting the idea that HCV proteins migrate to mitochondria by lateral diffusion from the ER *via* transient fusion of the membranous sub-compartments[34-36]. Although diverse, the molecular mechanisms that HCV adopts in disturbing mitochondria functions, generally concern redox state and calcium ($Ca^{2+}$) homeostasis. NS3 and E1 proteins, in fact, have been shown to induce enhanced ROS production and mitochondrial membrane potential ($mt\Delta\Psi$) reduction in a transient-expression system[37], as well as NS4A, that is able to provoke the release of cytochrome c from mitochondria into the cytosol[38] and NS5A, whose action on mitochondria most probably results from the drastic effects it produces on intracellu-

lar $Ca^{2+}$ signaling[39-41]. Taking up substantial amounts of $Ca^{2+}$ from the cytosol or the ER, mitochondria behave as large and dynamic $Ca^{2+}$ buffers and mitochondrial $Ca^{2+}$ uptake can be considered a real cellular mechanism which has a critical function in modulating physiological events like metabolic activity and cell fate[23,42]. The outward $Ca^{2+}$ flux from the ER to the mitochondria is facilitated by MAMs that provide local microdomains where the high localized $Ca^{2+}$ levels enable the functioning of the Deltapsi$_m$-driven $Ca^{2+}$ uniport at the inner mitochondrial membrane, which, together with the voltage-dependent anion channel (VDAC)/porin of the outer mitochondrial membrane (OMM), is mainly responsible for the entrance of $Ca^{2+}$ into the mitochondria[44,45]. The enhanced steady-state intramitochondrial $Ca^{2+}$ concentration has different functional consequences, among which the allosteric activation of tricarboxylic acid cycle (TCA cycle) and oxidative phosphorylation (OXPHOS) enzymes, resulting in an overall stimulation of respiratory chain (RC) activity and higher ATP output, is one of the best characterized[46,47]. However, if excessive, $Ca^{2+}$ accumulation by mitochondria could turn into a powerful trigger of the mitochondrial permeability transition pore (MPTP) permanent opening, resulting in osmotic swelling, breaking of the outer membrane, and finally, in the release of cytochrome c and other pro-apoptotic signalling molecules as a prelude to the programmed cell death activation[48]. Even though their relationship has not been completely clarified, several lines of evidence suggest that the directly induced oxidative stress is intimately connected to the alteration of mitochondrial $Ca^{2+}$ homeostasis provoked by HCV infection. The interplay between $Ca^{2+}$ and redox unbalance is far from being a linear cause-effect relationship, but novel outcomes resulting by our previous work[49] unequivocally identify the $Ca^{2+}$ overload in the mitochondrial compartment as the primary event leading to the profound mitochondrial oxidative metabolism alterations induced by the coordinated expression of all HCV proteins in a well-defined cellular context.

To the aim to gather and reorganize the complex and abundant available data on the subject and to delineate a possible sequence of molecular events following HCV infection a comprehensive mechanistic working model was recently proposed by our group[50].

It was proposed that the HCV-related outward flux of $Ca^{2+}$ from ER stores, following a direct interaction or an ER stress-mediated indirect effect of the virus[51-53], causes a reduction of the electrochemical transmembrane potential and thereby affects the efficiency of ATP synthesis. If timely limited, this effect may be counterbalanced by an adaptive stress-response consisting in the $Ca^{2+}$-mediated activation of TCA cycle and up-regulation of OXPHOS[45] but, in the case of a chronic viral infection, the persistent insult becomes very hard to counteract. High levels of mitochondrial $Ca^{2+}$ may in fact severely impair OXPHOS, provoking electron leakage to $O_2$ with formation of the superoxide anion $O_2^{\bullet -}$ over the basal level[54]. Since the main source of ROS, under these conditions, is the RC complex I that is also one of the major targets of superoxide, a kind of self-inhibition mechanism is established[32,49]. In addition, enhanced intramitochondrial $Ca^{2+}$ and ROS activate the MPTP whose opening causes flush out of low molecular weight metabolites comprising $NAD(P)^+$/NAD(P)H and the anti-oxidant molecule glutathione[55]. This further impairs the RC and the OXPHOS activity and reduces cellular ROS scavenging reserve with consequent worsening of the redox balance that strongly impacts on ER-mitochondria $Ca^{2+}$ homeostasis[56] and triggers a detrimental self-nourishing cycle.

The pathological outcomes of this profound HCV-related subversion of mitochondrial physiology strictly depend on the severity of ROS and mitochondrial $Ca^{2+}$-related insult and may also diverge in virus load or viral protein expression and/or in clinical features of the host. Under conditions of low ROS and $Ca^{2+}$-dependent stress level, a pro-survival and proliferative adaptive response is induced by redox signaling and, in spite of the impairment of the RC activity, ATP levels are even higher in HCV protein-expressing cells, as we reported in a previous work[57]. This apparently inconsistent phenomenon may be explained with the HCV-linked activation of the hypoxia-inducible factor 1 and the resultant shift of the energetic metabolism of infected cells toward glycolysis[57,58]. If low production of ROS is not damaging to the infected cell, it may in turns favor HCV maintenance and lead to accumulation of mutagenic hits resulting in carcinogenic priming of the host cell and ultimately in hepatocellular carcinoma development. Under conditions of high concentrations of mitochondrial $Ca^{2+}$ and ROS, instead, the MPTP permanent opening may lead, depending on the prevailing conditions, to selective removal of damaged organelles, apoptosis or necrosis[59]. If inadequate, apoptosis fails to remove cells carrying genetic alterations, promoting the development of hepatocellular carcinoma, that can be favored, at the same time, by chronic apoptotic stimulation and by the high rate of tissue regeneration induced that exposes cell to the risk of mitotic errors. However, the HCV struggle against mitochondria is not restricted to the injury of their cellular bioenergetic competence but it is also extended to hit the mitochondrial immune response. The recent identification of the MAVS protein (also known as CARDIF, VISA or IPS-1), which contains a C-terminal mitochondrial localization domain that targets it to the mitochondrial outer membrane, highlighted the so far underrated role of mitochondria in the antiviral innate immune response[60,61]. Recognition of early replication intermediates of HCV such as double-stranded RNA by the upstream sensors RIG-I and MDA5 induces a conformational change that results in their binding to MAVS that behaves as an essential adaptor propagating the signal downstream and finally inducing interferon-γ (IFN-γ) production[62]. Mitochondrial localization of MAVS is absolutely necessary for

its function, mistargeting of the protein away from mitochondria, in fact, completely abolishes this antiviral defense pathway[60]. Shortly after discovery of MAVS it was shown that the HCV NS3/NS4A complex avidly cleaves MAVS in a C-terminal anchor loop site, releasing the protein from the mitochondria. This mechanism enables HCV to paralyze the MAVS-downstream signalling pathway leading to IFNβ production, and to elude the host innate immunity in a continuously evolving fight for its survival[63].

## HCV AND AUTO/MITO-PHAGY: HOW A VIRUS CAN BENEFIT FROM A HOST DEFENSE MECHANISM

By putting into action stress relief responses, induction of cytokines and apoptosis, as well as by functioning as the powerhouses of the cell, mitochondria are key players in the life of a eukaryotic cell. Accordingly, it is not surprising that mitochondrial dysfunctions can also potentially damage cells and have been implied in a wide range of age-related disorders and various forms of cancer[64]. Accurate removal of functionally compromised mitochondria is, therefore, crucial to prevent cellular damage and to sustain cellular well-being. Thus, the finely regulated process of lysosomal-mediated bulk degradation of cytosolic components and organelles, including mitochondria, known as autophagy (from Greek, meaning "self-eating"), represents an important protection mechanism of the cell against stressful conditions, promoting cellular survival, differentiation, development and homeostasis[65]. Originally characterized as an evolutionarily conserved cellular response to nutrient starvation, this "self-digestion" pathway not only contributes to maintain vital cellular functions in fasting conditions by mobilizing nutrients from macromolecular degradation, but also can rid the cell of unnecessary or damaged organelles, protein aggregates and even invading microorganisms[66]. Three types of autophagy, *i.e.*, macroautophagy, microautophagy, and chaperone-mediated autophagy, have been described to date, even if macroautophagy is the most extensively studied autophagy pathway that mediates the large-scale degradation of intracellular molecules. In the initial steps of this process, cytoplasmic material is engulfed by an isolation membrane, which is also called phagophore whose edges then fuse to form the double-membraned vesicles named autophagosomes. This is followed by fusion of the autophagosome with a lysosome to form an autolysosome where the captured material, together with the inner membrane, is degraded[67]. In addition to the master regulator of autophagy, the target of rapamycin, TOR kinase, which acts by inhibiting autophagy in response to insulin-like and other growth factor signals, other regulatory molecules of autophagy including Bcl-2, ROS, $Ca^{2+}$, and the AMP-activated protein kinase have recently been reported. In addition, more than 30 autophagy-related proteins, Atg, have been identified to date, in yeast, many of which are evolutionarily conserved, such as the mammalian Atg6/Vps30 ortholog Beclin 1[68,69]. The core machinery of macroautophagy and the various steps of this process are similar whether invoked for the clearance of bulk cytosol or for the selective elimination of mitochondria that are specifically removed by a dedicated pathway called "mitophagy[70]. Whether serving as a quality control mechanism to eliminate harmful damaged mitochondria, maintaining mitochondrial functional and genetic integrity, or to modulate the steady-state turnover of mitochondria and their number in response to developmental or physiological cues, mitophagy has established itself as an increasingly important process[71]. In many metazoan cell types, mitophagy is mediated by a pathway comprised of PTEN-induced putative protein kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin, whose defects are associated with a form of autosomal recessive juvenile Parkinson's disease. Pink1 and Parkin physically interact with each other and, as suggested by genetic studies, Pink1 is upstream of Parkin. Pink1/Parkin pathway is triggered when PINK1, accumulated on the outer membrane of damaged mitochondria, facilitates recruitment of cytosolic Parkin, promoting mitochondria segregation from the mitochondrial network and targeting these organelles for their mitophagic clearance[70,72,73]. In mammalian cells, Parkin normally resides in the cytosol but it is selectively recruited to depolarized mitochondria, promoting the colocalization of mitochondria with the autophagy marker LC3[74]. How does Parkin promote mitophagy? Emerging evidence has suggested that Parkin plays a dual role in mitophagy process, priming the mitochondria through the ubiquitination of OMM proteins on the depolarized mitochondria[75], and promoting induction of autophagy by interacting with Ambra1 and activating class Ⅲ PI3K[76]. Initially supposed to be exclusively dedicated to the "recycling" of macromolecular material within the cell, the autophagy machinery is now emerging as a process that interfaces with most cellular stress-response pathways, including those involved in controlling immune responses and inflammation[77,78]. The immunological role of autophagy is, in fact, a newly recognized facet of innate and adaptive immunity against viral infection and certain viruses have developed strategies to counteract these antiviral mechanisms while others have become even able to co-opt the autophagy machinery as a proviral host factor favoring their own survival[79]. Recognition of viral RNA by innate immune sensors, as well as engagement of CD46, a cell surface receptor required for entry of a variety of pathogens[80], are only some of the different mechanisms of autophagy induction in infected cells. Liang *et al*[81] were the first to demonstrate the antiviral potential of autophagy by showing that the overexpression of Beclin1 by a recombinant Sindbis virus (SV) decreases SV replication and SV-induced neuronal apoptosis, protecting mice from fatal SV encephalitis. The innumerable strategies that viruses,

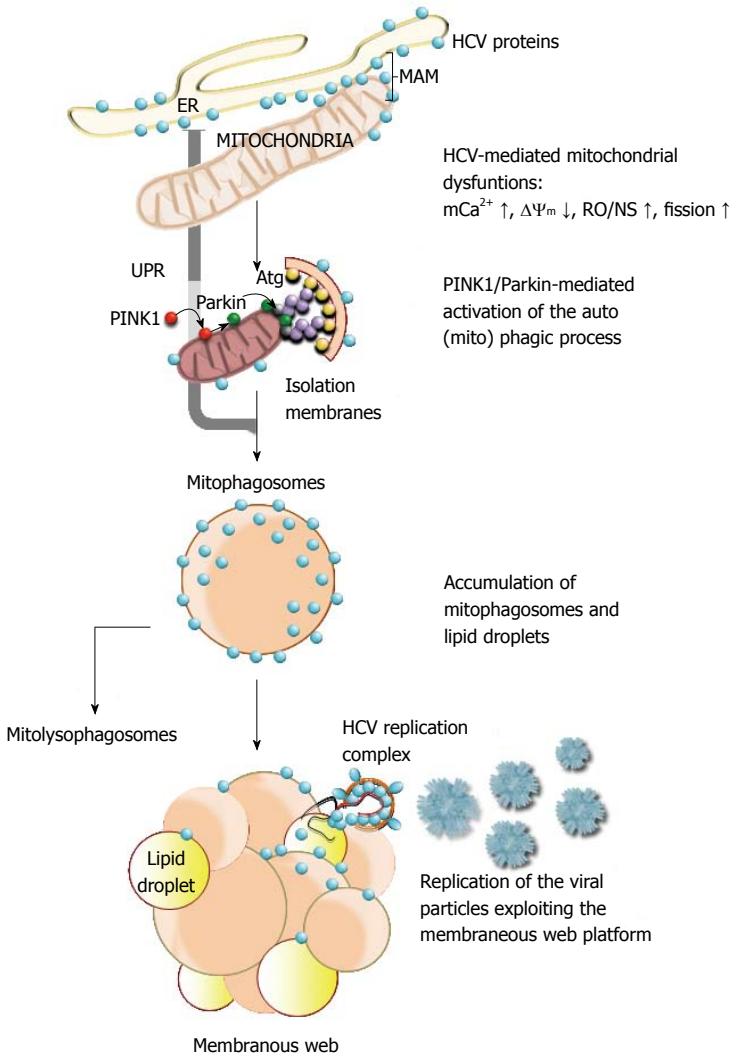


**Figure 1 Schematic representation of the hepatitis C virus-induced auto(mito)phagy in infected host cell.** The cartoon illustrates the suggested sequential steps leading to activation of the macro-autophagic process by the hepatitis C virus (HCV) proteins, which are shown as pale-blue circles localized at the endoplasmic reticulum (ER) membranes where translation and proteolytic processing of the HCV polyprotein take place. Through the mitochondria associated membranes (MAMs) the HCV proteins partly transfer to mitochondrial membranes inducing therein a number of alterations comprising enhanced influx of $Ca^{2+}$, decrease of the respiratory chain activity and of the transmembrane potential, increase of reactive oxygen/nitrogen species (RO/NS), promotion of the mitochondrial network fragmentation (fission). The dysfunctional mitochondria recruit on the their outer membrane the kinase PTEN-induced putative protein kinase 1 (PINK1), which phosphorylates and activates the ubiquitin ligase Parkin. Ubiquitinated mitochondrial proteins target the organelle to the nascent macrophagic vescicles (isolation membranes) through interaction with autophagy-related gene (Atg) factors thereby leading to progressive engulfment of mitochondria in the auto(mito)phagosomes. If the rate of macrophagosomes formation overwhelms that of their degradation, *via* fusion with lysosomes (mitolysophagosomes), this leads to accumulation of macrophagic vesicles that combines to lipid droplets developing a membranous web. This provides a structural/functional platform where the HCV replication complex assembles and releases viral particles. Although the scheme highlights a major role of the HCV protein-mediated mitochondrial dysfunctions in the induction of the autophagic process the participation of the HCV protein-induced ER stress-mediated unfolded protein response (UPR) is also shown. See text for further explanations and references.

as well as bacteria, developed to neutralise the host autophagic defense mechanisms highlight the importance of this process in immunity. These include the blockade of positive upstream regulators of autophagy, such as the IFN-inducible RNA-activated eIF2α protein kinase (PKR) signalling pathway, the activation of negative upstream regulators of autophagy, such as the nutrient-sensing TOR kinase signalling pathway or direct antagonism of the autophagy machinery. As mentioned above, certain viruses might manipulate the autophagic pathway or at least specific autophagy genes to foster their own self-serving purposes. It is the case of poliovirus, rotavirus, HIV, coronaviruses, dengue virus, and the hepatitis B and C viruses, which exploit autophagy proteins for their membrane formation and/or trafficking functions[82]. Interestingly, as cellular membranous structures, autophagosomes have been proposed to act as a scaffold for intracellular membrane-associated replication of certain cytoplasmic RNA viruses[83]. Actually, it has been demonstrated that negative inhibition and down-regulation of different regulators of the autophagy pathway strongly suppress productive HCV infection. Autophagy proteins (*i.e.*, Beclin-1, Atg4B, Atg5, and Atg12) behave, in fact, as proviral factors, required to initiate translation of the

incoming HCV RNA in *de novo* infected cells, but they are not required once infection is established[84]. Interestingly, several independent studies suggest that HCV is able to induce a cellular ER stress response, also termed the unfolded protein response (UPR), inducing the accumulation of autophagosomes in cells without enhancing autophagic protein degradation. Therefore, autophagosomes turn into sanctuaries for HCV replication and protection from host immune surveillance that favour chronic infection and liver injury. The inhibition of UPR signaling pathways in fact suppresses HCV-induced lipidation of LC3 protein, a necessary step for the formation of autophagosomes, suggesting a positive role of UPR and the partial autophagic response in HCV replication[85-87]. Moreover, Chu *et al*[88], also showed that HCV-induced ROS production, both in the cytosol and mitochondria of HCV protein-expressing hepatoma cell lines might contribute to the activation of autophagy.

Considering the above-mentioned multitude of HCV-induced pathophysiological insults leading to mitochondrial dysfunction, it might sound unsurprising that HCV also affects mitophagy. The first demonstration of the direct effect of HCV on a key mechanism responsible for mitochondrial turnover and quality control resulted from very recent studies by Kim *et al*[89], that revealed the ability of HCV to induce Parkin-dependent mitophagy. Using multiple strategies including confocal and electron microscopy, the authors observed a striking phenomenon of clustering of mitochondria in the perinuclear regions of the infected cells associated to mitochondrial translocation and aggregation of Parkin, irrespective of HCV genotypes. They also proved that HCV infection enhances Parkin-mediated ubiquitination of its known substrates Mfn2 and VDAC1 and that this process is attenuated by Parkin silencing. Furthermore, it was also shown that HCV infection induces an increase in both mRNA and protein levels of Parkin and PINK1. Significantly, increased Parkin protein levels were also found in liver tissues samples obtained from chronic HCV patients. An enhanced Parkin-mediated mitophagosome formation process also characterized HCV-infected cells in comparison to uninfected cells, followed by their later delivery to lysosomes to originate mitophagolysosomes. Once again, therefore, HCV usurps a physiological cell function to its own purposes, but which are these purposes? Kim *et al*[89] also highlighted that Parkin and PINK1 silencing negatively affects HCV RNA replication, suggesting a possible role of mitophagy in the viral life-cycle (Figure 1). Finally, it was demonstrated that the above-mentioned HCV-induced decrease in mitochondrial complex I activity, is surprisingly reversed by knockdown of Parkin that, similarly, also restores the number of mitochondria, usually reduced in HCV infected cells. Based upon these observations, the authors proposed a functional involvement of HCV-induced mitophagy in the impairment of oxidative phosphorylation and depletion of mitochondria that HCV typically provokes in the host cells.

## CONCLUSION

Despite large research effort, knowledge about the intersection between the auto/mitophagy pathway and HCV infection is still quite rudimentary. However, among the diverse and complex physiological processes that can negatively or positively impact the course and natural history of the HCV-infection and the survival of both virus and host, these fundamental quality control systems undoubtedly play a pivotal role. Recent advances about autophagy functions are in fact reshaping our understanding of the pathogenesis of infective diseases. Since, further detailed analyses of the molecular mechanisms whereby virus exploits, for its benefit, the various components of the same auto/mitophagy machinery on which host relies to defend itself will greatly enhance our understanding of HCV associated liver disease pathogenesis and possibly, lead to the design of new selective antiviral therapeutic approaches.

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# Serrated pathway in colorectal carcinogenesis

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## Abstract

Serrated adenocarcinoma is a recently described subset of colorectal cancer (CRC), which account for about 10% of all CRCs and follows an alternative pathway in which serrated polyps replace the traditional adenoma as the precursor lesion to CRC. Serrated polyps form a heterogeneous group of colorectal lesions that includes hyperplastic polyps (HPs), sessile serrated adenoma (SSA), traditional serrated adenoma (TSA) and mixed polyps. HPs are the most common serrated polyp followed by SSA and TSA. This distinct histogenesis is believed to have a major influence in prevention strategies, patient prognosis and therapeutic impact. Genetically, serrated polyps exhibited also a distinct pattern, with KRAS and BRAF having an important contribution to its development. Two other molecular changes that have been implicated in the serrated pathway include microsatellite instability and the CpG island methylator phenotype. In the present review we will address the current knowledge of serrated polyps, clinical pathological features and will update the most recent findings of its molecular pathways. The understanding of their biology and malignancy potential is imperative to implement a surveillance approach in order to prevent colorectal cancer development.



**Key words:** Serrated pathway; Colorectal carcinogenesis; Mutation; Microsatellite instability; CpG island methylator phenotype

**Core tip:** This paper reviews the pathologic and molecular features of serrated polyps and the serrated pathway to colorectal cancer and its clinical impact. The serrated pathway has recently emerged as the second pathway leading to colorectal cancer, and the genetic alterations occurring in this pathway are not still clarified. It's imperative to understand the molecular profile of colorectal lesions with higher malignancy potential to implement a surveillance and screening approach in order to prevent colorectal cancer development.

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## INTRODUCTION

Colorectal cancer (CRC) is the third most frequent cancer worldwide, with more than one million incident cases and is the fourth most common cause of cancer deaths accounting for approximately 609000 deaths[1]. In

**Table 1 Frequency of hyperplastic polyps subtypes *n* (%)**

| Author | Population | HPs, *n* | MVHP | GCHP | MPHP |
|---|---|---|---|---|---|
| Kim *et al*[24] | South Korea | 45 | 30 (66.7) | 11 (24.4) | 4 (8.9) |
| Carr *et al*[18] | Australia | 36 | 34 (94.4) | 2 (5.6) | 0 (0.0) |
| Spring *et al*[27] | Australia | 120 | 54 (45.0) | 66 (55.0) | 0 (0.0) |

HP: Hyperplastic polyp; MVHP: Microvesicular hyperplastic polyp; GCHP: Globet cell hyperplastic polyp; MPHP: Mucin-poor hyperplastic polyp.

the classical genetic model for colorectal tumorigenesis described by Fearon and Vogelstein[2] the evolution of colorectal cancer follows the adenoma-adenocarcinoma sequence, which is driving by the progressive accumulation of a number of critical mutations[2]. In this model the adenomatous polyp is the principal precursor of colorectal cancer[2,3]. More than 90% of colorectal cancers are adenocarcinomas and subtypes include medullary, micropapillary, mucinous, serrated and signet ring cell[4]. Serrated carcinomas were first described by Jass and Smith[5] and represents the progression of a dysplastic serrated lesion, most commonly serrated adenomas. Serrated adenocarcinomas accounts for about 10% of all CRCs, and follows an alternative pathway in which serrated polyps replace the traditional adenoma as the precursor lesion to colorectal cancer[6]. Serrated polyps form an heterogeneous group of colorectal lesions that includes hyperplastic polyps (HPs), sessile serrated adenoma (SSA), traditional serrated adenoma (TSA) and a combination of two or more characteristics, formerly classified as mixed polyps (MP)[4,7-9]. This distinct histogenesis is also believed to have a major influence in prevention strategies, patient prognosis and therapeutic impact[6,10-12].

Molecularly, the classical adenoma-carcinoma sequence pathway is mainly governed by chromosomal instability (CIN) and *KRAS* mutations[3], whereas in the serrated pathway the genetic alterations include *BRAF* mutation and gene promoter hypermethylation (CpG island methylator phenotype or CIMP)[13]. Microsatellite instability (MSI) is another molecular pathway that can be detected in both the adenoma-carcinoma sequence and the serrated pathway[14,15].

Morphologically colorectal carcinomas that harbors CIN and arises from adenoma have a classical histological feature of dirty necrosis[6]. The carcinomas with microssatelite instability generally occurs in the right colon, are mucinous or poorly differentiated (medullary histology) and also have intraepithelial lymphocytes and lymphoid aggregates "Crohn like"[4]. The serrated carcinomas that originate from traditional serrated adenomas generally are MSS or MSI-L and those that originate from a sessile serrated adenoma are MSI-H. Some histological features are typically found and used to classify colorectal carcinoma as serrated carcinoma. The most important histological features are: presence of epithelial serrations, clear or eosinophilic cytoplasm, abundant cytoplasm, vesicular nuclei, absence of necrosis, mucin production and presence of cell balls and rods. Other very important histological finding that helps to make a diagnostic of serrated carcinoma is the presence of serrated lesion in the periphery of the infiltrative carcinoma[6].

Due to this well recognized step-wise progression of premalignant lesions to carcinomas, CRC has a particular and outstanding feature in that make it amenable to prevention strategies with detection of removal of those susceptible lesions[16]. Therefore, it is imperative to understand the lesions with higher malignancy potential and with the use of their molecular profile to implement a surveillance approach in order to prevent colorectal cancer development[17].

In this article, we review the pathologic and molecular features of serrated polyps and the serrated pathway to colorectal adenocarcinoma.

## *Morphological aspects of serrated polyps*

The serrated polyps are characterized by the serrated morphology, hypermaturation of the gland epithelium due to low extent of the cell loss by apoptosis[18,19]. The classification of the serrated lesions by a pathologist is based mainly on architectural criteria like growth pattern, cytological dysplasia and serration of the crypts[4,8,19-21] (Figure 1). The reliable classification of serrated polyps is fundamental to surveillance of patients with these precursor lesions[4]. Yet, this is difficult task, due to the problems with accurate histological definition, leading to a high inter-observer variation, even among expert pathologists[4,8,13,19,21,22].

HPs are the most common serrated polyp of the colon accounting for about 80% to 90% of all serrated polyps and around 10% to 15% of all polyps of the colon. HPs are generally small (< 5 mm) and frequently located in the distal colon (75%-80% in the rectosigmoid)[6,23]. According to the World Health Organization, three subtypes of HP have been recognized: the microvesicular (MVHP), goblet-cell rich hyperplastic polyps (GCHP) and mucin-poor types (MPHP). The differences between them are based mainly in morphology and on the cellular mucin distribution[4,13,24]. All HPs subgroups are histopathology distinguished by elongation of the crypts with different degrees of serration[4,25] and tend to have no dysplasia[8]. MVHP, the most frequent subtype, is characterized by epithelial cells with vesicular mucin and decrease in the goblet cells, concomitantly with conspicuous serration often located in the basal portion of the crypts[4,13,24]. On the other hand, the GCHP has many mature cells in the upper crypt with subtle serration[4,13,26]. The MVHP is often seen in the right colon, whereas the GCHP is more frequently observed in the left-sided colon. The MPHP is rare and therefore, less frequently discussed. It seems to be more frequent in the left colon[6]. There are still great divergences about the frequency of each HP subtypes (Table 1). Spring *et al*[27] reported that MVHP was observed in 45% and GCHP in 55% of 120 hyperplastic polyps in the unselected series of 190 patients and 414 lesions. Recently, Kim *et al*[24] related that MVHP, GCHP and MPHP accounted with 66.7%;

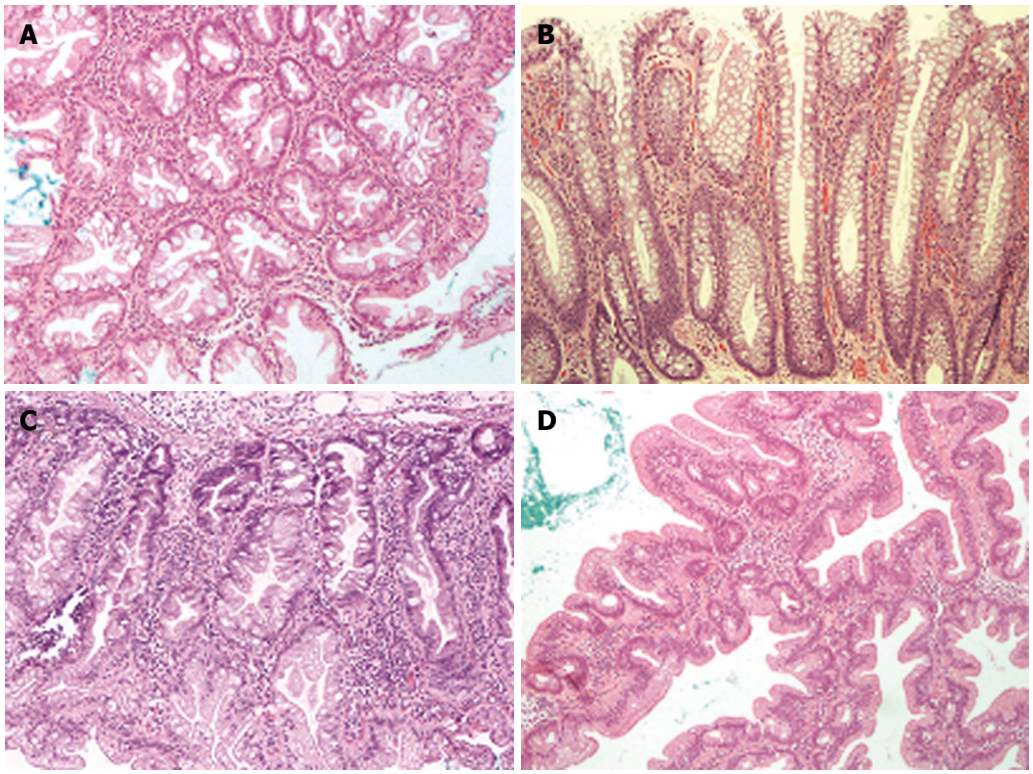


**Figure 1 Hematoxylin and eosin of representative cases of serrated lesions.** A: Microvesicular hyperplastic polyp (× 100); B: Goblet cell hyperplastic polyp (× 100); C: Sessile serrated adenomas (× 100); D: Traditional serrated adenomas (× 100).

24.4% and 8.9%, respectively, of the 45 HPs studied. On the other hand, Carr *et al*[18] found only 2 (5.6%) GCHPs among 34 HPs studied. Despite these divergences, the clinical relevance in the recognition of the HPs subtypes is still indefinite, suggesting that more studies are needed to clarify this aspect[13,25].

The SSAs account for about 3%-9% of all the colorectal polyps[18,21,27] and 10%-25% of all serrated polyps[4,26,27] and are generally located in the right colon. The TSAs are infrequent lesions, located predominantly in the left colon and account for about 1%-2% of the serrated lesions[18,27]. The histopathology characteristics of the SSA includes dilatation of the base of the crypts which often grow parallel to the muscularis mucosa forming L shaped or inverted T-shaped crypts (*anchor form*)[4,13,19]. SSAs have elongation of crypts with prominent serration[4,7,27]. These lesions might have subtle nuclear atypia, where mitoses may be seen in the crypts. Since the SSA has similarities with MVHP the classification is made based mainly in the analysis of the crypts: if two or three adjacent crypts demonstrate features of SSA, it must be classified into SSA[4]. When conventional cytological dysplasia is observed, the serrated polyps are classified as TSA[18]. Nonetheless, due to the similarities with conventional adenomas, it is recommended that this term never be used without qualifier[4]. The TSA has overall complex and villiform growth pattern, showing cells with cytological dysplasia that may indicates progression to carcinoma. TSA differ from SSA mainly because TSA lose the anchoring leading the formation of ectopic crypts[4,18]. Further, it is rare to observe mitosis in TSA and columnar cells with eosinophilic cytoplasm are features of these lesions[4,13].

MPs account for about 0.7%-1.5% of all colonic polyps and for 1.7% to 4.7% of the serrated polyps[18,27,28]. Mixed polyps combine at least two characteristics of conventional adenomas, SSA, TSA or HPs[18,21,28-30]. The main feature of MP is the combination of nondysplastic polyps (HP or SSA) with the dysplastic one (TSA or conventional adenomas)[21,27].

### *Serrated polyps and natural history of CRC: evidence linking serrated polyps with CRC*

In the classic adenoma-carcinoma sequence model of colorectal tumorigenesis proposed by Fearon and Vogelstein, HPs were described as harmless non neoplastic lesions with no malignant potential[2,24,31]. Though, a new understanding of the pathology and natural history of CRC has emerged over the past decade. Approximately, 10% of sporadic colorectal cancers, named "serrated adenocarcinoma" will arise *via* serrated polyp-carcinoma sequence[11]. In this context, hyperplastic polyps were recently recognized as neoplastic lesions included in the serrated group and may predispose to cancer. In this new model, HPs may progress to other serrated polyp including sessile serrated adenomas, traditional serrated adenomas or mixed polyps and then evolve to colorectal cancer[32]. It has been estimated that HPs take 7.5 years to progress to serrated adenoma[33]. However, only a tiny percentage of hyperplastic polyps will progress to cancer[30,31,34,35]. Large and often right-sided HPs are more

**Table 2 Description of molecular alterations reported in serrated lesions**

| Ref. | Population | Molecular alterations | Serrated polyp | | | | | | Carcinoma |
|---|---|---|---|---|---|---|---|---|---|
| | | | HP | | | SSA | TSA | MP | Serrated ADC |
| | | | MVHP | GCHP | MPHP | | | | |
| Kim *et al*[24] | South Korea | *KRAS* | 16.7% | 72.7% | 25.0% | 12.5% | NA | NA | NA |
| | | *BRAF* | 66.7% | 0.0% | 25.0% | 60.7% | NA | NA | NA |
| | | MSI-H | NA | NA | NA | 1.8% | NA | NA | NA |
| | | CIMP positive | 73.3% | 18.2% | 75.0% | 76.8% | NA | NA | NA |
| Sandmeier *et al*[30] | Switzerland | *KRAS* | | 17.0% | | 25.0% | NA | NA | NA |
| | | *BRAF* | | 83.0% | | 63.0% | NA | NA | NA |
| | | MSI-H | | 0.0% | | 0.0% | NA | NA | NA |
| | | CIMP positive | | NA | | NA | NA | NA | NA |
| Kim *et al*[10] | United States | *KRAS* | 6.0% | 8.0% | NA | 8.0% | 17.0% | 25.0% | NA |
| | | *BRAF* | 88.0% | 75.0% | NA | 81.0% | 76.0% | 75.0% | NA |
| | | MSI-H | 0.0% | 0.0% | NA | 0.0% | 3.0% | 0.0% | NA |
| | | CIMP positive | 41.%% | 8.0% | NA | 44.0% | 43.0% | 50.0% | NA |
| O´Brien *et al*[40] | United States | *KRAS* | 13.2% | 42.9% | NA | 6.9% | NA | NA | 0.0% |
| | | *BRAF* | 76.3% | 21.4% | NA | 82.9% | NA | NA | 82.0% |
| | | MSI-H | 0.0% | 0.0% | NA | 0.0% | NA | NA | 81.8% |
| | | CIMP positive | 47.4% | 14.3% | NA | 75.9% | NA | NA | 90.0% |
| Spring *et al*[27] | Australia | *KRAS* | 11.0% | 50.0% | NA | 8.0% | 0.0% | 43.0% | NA |
| | | *BRAF* | 70.0% | 20.0% | NA | 78.0% | 66.0% | 57.0% | NA |
| | | MSI-H | NA | NA | NA | NA | NA | NA | NA |
| | | CIMP positive | NA | NA | NA | NA | NA | NA | NA |
| Konishi *et al*[34] | Japan | *KRAS* | | 13.0% | | 8.0% | NA | 22.7% | NA |
| | | *BRAF* | | NA | | 32.0% | NA | 40.9% | NA |
| | | MSI-H | | 8.0% | | 36.0% | NA | 5.0% | NA |
| | | CIMP positive | | NA | | NA | NA | NA | NA |
| Yang *et al*[33] | United States | *KRAS* | 13.2% | 46.2% | NA | 7.1% | 28.0% | NA | NA |
| | | *BRAF* | 76.3% | 23.1% | NA | 82.1% | 60.0% | NA | NA |
| | | MSI-H | NA | NA | NA | NA | NA | NA | NA |
| | | CIMP positive | 47.4% | 15.4% | NA | 75.0% | 80.0% | NA | NA |

HP: Hyperplastic polyps; MVHP: Microvesicular hyperplastic polyp; GCHP: Globet cell hyperplastic polyp; MPHP: Mucin-poor hyperplastic polyp; SSA: Sessile serrated adenoma; TSA: Traditional serrated adenoma; MP: Mixed polyp; MSI-H: High microsatellite instability; CIMP: CpG island methylator phenotype; ADC: Adenocarcinoma; NA: Not applicable.

likely to have malignant potential[35-37].

At the genetic level, there are also evidences showing that serrated polyps are strongly associated with the development of colorectal neoplasms, as further discussed.

### *Putative genetic pathways in serrated carcinomas*

Serrated pathway has recently emerged as the second pathway leading to colorectal cancer, therefore, the genetic alterations occurring in this pathway are not clarified and there is great variability in the frequency of molecular changes described. Results of recent studies reporting genetic analysis in the serrated lesions are summarized in Table 2. A schematic view of serrated polyps-carcinoma sequence is shown in Figure 2.

The most frequent genetic alterations involve *BRAF* and *KRAS* mutations. Both *KRAS* and *BRAF* encodes kinases that belong to the mitogen-activated protein kinase (MAPK) cascade that mediates the cellular signaling involving cell proliferation, apoptosis and differentiation[38]. Mutations in *KRAS* and *BRAF* oncogenes result in the constitutive activation of the MAPK pathway and in uncontrolled cell proliferation, cell survival, invasion and metastisation[38]. Mutations in both oncogenes are frequently found as mutually exclusive events in serrated adenocarcinoma[39] and in the precursor serrated lesions[33]. Stefanius *et al*[39] demonstrated a high frequency of *KRAS* mutations (45.2%) in serrated adenocarcinoma, suggesting that a significant proportion of *KRAS* mutated CRC originates from serrated polyps. O´Brien *et al*[40] showed high frequency of *BRAF* mutation (V600E) among serrated carcinomas (82%), emphasizing that this mutation is a specific marker in the serrated pathway.

*KRAS* mutations occur predominantly at codon 12 and less frequently at codon 13, and the most common mutations are G12D, G12V and G13D[27], being mutated in 0%-73% of serrated polyps[6,10,27,30,33,34,40], 6%-73% of HPs, 7%-25% of SSA[10,24,27,30,33,34,40], and in 0%-28% of TSA[10,27] (Table 2). These codons are also the most frequent mutated in colorectal cancer[41]. Concerning *BRAF*, the most frequent mutation is V600E which occur in 0%-88% of HPs, 32%-82.9% of SSA, and in 60%-76% of TSA[24,27,33,34,40,42] (Table 2). *BRAF* mutations are more frequent than *KRAS* mutations in MVHP and SSA (Table 2)[24,27,33,34,40]. On the other hand, in GCHP, *KRAS* mutations are likely the most important genetic alteration. One study, by Kim *et al*[10] showed higher frequencies of *BRAF* mutations than *KRAS* mutation in both MVHP and GCHP.

Another molecular alteration described in serrated lesions is MSI, a hallmark of colorectal cancer arising

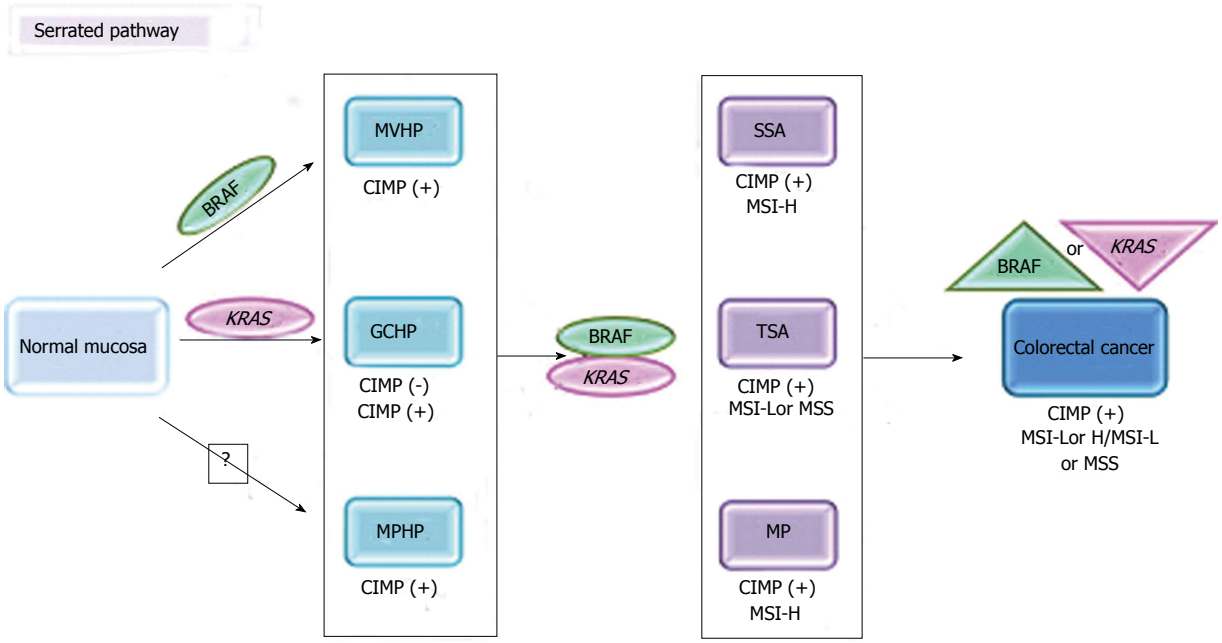


**Figure 2 Schematic view of serrated polyps: Carcinoma sequence.** MVHP: Microvesicular hyperplastic polyp; GCHP: Globet cell hyperplastic polyp; MPHP: Mucin-poor hyperplastic polyp; SSA: Sessile serrated adenoma; TSA: Traditional serrated adenoma; MP: Mixed polyp; MSI-H: High microsatellite instability; CIMP: CpG island methylator phenotype.

in the context of Hereditary Non Polyposis Colorectal Cancer or Lynch syndrome[43] (Table 2 and Figure 2). The MSI is caused by the lost of mismatch-repair genes[43], which leads to an increased susceptibility to accumulate mutations in genes with microsatellite regions[43]. The MSI status can be classified according to the markers that show instability[34]. It was proposed a panel of five microsatellites sequencing, known as Bethesda panel, for identify the MSI status[4,10,11,44]. This panel include two mononucleotide markers (BAT 25 and BAT 26) and three dinucleotide microsatellites (D5S346, D2S123 and D17S250), but three other mononucleotide markers can be included (NR21, NR22 and NR24) in a pentaplex PCR[45]. Tumors that have two or more unstable markers are considered high MSI (MSI-H), when only one marker is unstable tumors are defined as low MSI (MSI-L)[4,10,11,44], and tumors are defined as microsatellite stable (MSS), when no instability is identified at those five loci[4,11,44]. About 15%-20% of sporadic colorectal cancers are MSI-H. Importantly, MSI-H tumors exhibited a distinct genetic pathway of the MSS and MSI-L tumors, without major chromosomal alterations, but showed the presence of mutations in genes with microsatellite regions, known as MSI-target genes[4,24,44]. Stefanius *et al*[39] reported that 20.6% of serrated cancer showed MSI-H. Studies show that the precursor serrated lesions rarely demonstrate high levels of MSI (Table 2). Kim *et al*[10], observed that MSI-H was presented in 3.0% of TSA (Kim, 2008). Contrastingly, Konishi *et al*[34] reported that MSI-H was observed in 36% of sessile serrated adenomas (Table 2).

The CpG island methylator (CIMP) phenotype is also strongly related to the colorectal serrated carcinogenesis[24,44] (Table 2 and Figure 2). The methylation in the CpG island may cause transcriptional silencing, and inhibits gene expression by the binding of methyl groups to recurrent cytosine-guanine dinucleotides sequences, commonly in promoter region. This is an epigenetic event observed in the precursor serrated lesions and colorectal polyps[4,10]. The CIMP is frequent in serrated polyps mainly in the proximal colon[13]. The phenotype of CpG island methylator in hyperplastic polyps account for 41.0%-73.3% of MVHP, 8.0%-18.2% of GCHP and 75% of MPHP[24,33] (Table 2). Among serrated adenomas, CIMP-H is frequently observed in 44.0%-76.8% of SSA[24,33,40] and in 43%-80% of TSA[10]. Aberrant hypermethylation of CpG island is more frequently associated with *BRAF* mutation than with *KRAS* mutation in serrated cancers. It is frequently described that serrated lesions with *KRAS* mutation demonstrate low levels of CIMP distinctly to *BRAF* serrated lesions, often characterized by CIMP-H (Figure 2 and Table 2). High frequency of methylation was associated with polyps with large size that are > 1 cm and with high-grade dysplasia[10]. The status of CIMP is also often correlated with MSI status and mutations in both, *KRAS* and *BRAF* oncogenes[3,4,8,29]. According to Sandmeier *et al*[30], *BRAF* mutations were associated with *MLH1* and/or *p16* methylation in 88% of right-sided SSAs.

### *Clinical impact of the serrated pathway*

Understanding of natural history and malignant potential of colorectal polyps is essential for management of these lesions. After complete removal of adenomatous polyps, surveillance recommendations are well established based on risk for subsequent adenomas[14]. On the other hand, there is still lack of studies of follow up intervals of ser-

rated polyps and more accurate information about management of serrated polyps of the colon is not yet available[17,23]. The great majority of serrated polyps will never progress to carcinoma, nevertheless, it has been described that some patients harboring mainly sessile serrated lesions larger than 10 mm have increased risk to develop neoplasia. Therefore, despite absence of controlled studies TSA and SSA have been included among the lesions requiring surveillance[23]. Detecting and removing theses lesions may contribute to the prevention of colorectal cancer arising *via* the serrated pathway.

In addition, there is also growing evidence that cancers arising through the serrated pathway may differ from cancers arising through adenoma pathway in their prognosis and response to therapy. Serrated adenocarcinoma is likely to have a less favorable 5-year survival than conventional cancers[6,12]. The differences in the MSI status in the serrated adenocarcinomas may have therapeutic implications, which require that the patient be followed carefully[6,46,47].

The knowledge of serrated pathway during colorectal carcinogenesis represents a clinical challenge in the surveillance of patients harboring serrated polyps. Despite of gaps in our knowledge about biological behavior of serrated polyps, the molecular alterations reported so far, has allowed the understanding of serrated carcinogenesis and paving the way for future direction in CRC prevention.

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ORIGINAL ARTICLE

# Sodium alginate ameliorates indomethacin-induced gastrointestinal mucosal injury *via* inhibiting translocation in rats

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## Abstract

**AIM:** To investigate the effects of sodium alginate (AL-Na) on indomethacin-induced small intestinal lesions in rats.

**METHODS:** Gastric injury was assessed by measuring ulcerated legions 4 h after indomethacin (25 mg/kg) administration. Small intestinal injury was assessed by measuring ulcerated legions 24 h after indomethacin (10 mg/kg) administration. AL-Na and rebamipide were orally administered. Myeloperoxidase activity in the stomach and intestine were measured. Microvascular permeability, superoxide dismutase content, glutathione peroxidase activity, catalase activity, red blood cell count, white blood cell count, mucin content and enterobacterial count in the small intestine were measured.

**RESULTS:** AL-Na significantly reduced indomethacin-induced ulcer size and myeloperoxidase activity in the stomach and small intestine. AL-Na prevented increases in microvascular permeability, superoxide dismutase content, glutathione peroxidase activity and catalase activity in small intestinal injury induced by indomethacin. AL-Na also prevented decreases in red blood cells and white blood cells in small intestinal injury induced by indomethacin. Moreover, AL-Na suppressed mucin depletion by indomethacin and inhibited infiltration of enterobacteria into the small intestine.

**CONCLUSION:** These results indicate that AL-Na ameliorates non-steroidal anti-inflammatory drug-induced small intestinal enteritis *via* bacterial translocation.



**Key words:** Sodium alginate; Non-steroidal anti-inflammatory drugs; Gastrointestinal mucosal injury; Mucin; Bacterial translocation

**Core tip:** Sodium alginate ameliorates small intestinal enteritis *via* bacterial translocation.

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## INTRODUCTION

It is well-known that non-steroidal anti-inflammatory drugs (NSAIDs) damage the stomach. Small bowel injuries from these drugs have also been reported. Patients

who are on long-term NSAIDs treatment develop mucosal injuries of the small intestine, including bleeding, erosion, and ulcers[1-4]. In rats, indomethacin, one of the most commonly used NSAIDs, causes significant gastrointestinal damage[5,6], and several enteropathic consequences have been identified, including bacterial translocation[7], neutrophil activation[8], oxidative stress[9], and mucin deficiency[10].

Proton pump inhibitors (PPIs) significantly improve NSAIDs-induced pathologies of the gastrointestinal tract[11]. However, PPI may not demonstrate a therapeutic effect on NSAIDs-induced small bowel disease because gastric acid contributes little to small intestinal ulceration[12,13]. Moreover, it has been reported that PPIs exacerbate NSAIDs-induced small intestinal injury in rats by causing dysbiosis[14]. Therefore, drugs that protect against NSAID-induced gastrointestinal injury are needed. Rebamipide, a mucosal protective agent, has been reported to suppress NSAIDs-induced intestinal injury[15,16]. However, the development of a better therapeutic agent is needed. It was reported that rebamipide, one of mucosal protective agent, suppressed NSAIDs-induced intestinal injury[15,16]. But, the development of more therapeutic agent is demanded.

Sodium alginate (AL-Na) is a polysaccharide with homopolymeric blocks of (1-4)-linked β-D-mannuronate, and its C-5 epimer α-L-glucuronate residues are widely distributed in the cell walls of brown algae. AL-Na elicits a muco-protective effect by covering the surface of the gastrointestinal tract. Therefore, it may be useful for the treatment of gastric and esophageal ulcers and bleeding[17-22]. Recently, it was reported that AL-Na may be an efficacious, non-erosive treatment for reflux disease[23], and it may also be a useful in the treatment of upper gastrointestinal disease. Moreover, Humphreys *et al*[24] reported that AL-Na was poorly absorbed, primarily through the gastrointestinal tract, and was excreted in the feces. Therefore, AL-Na may be effective in both the upper and lower gastrointestinal tracts. Additionally, AL-Na is reportedly an effective treatment for experimental colitis[25,26] and for radiation-induced colon damage[27,28]. Therefore, we hypothesised that AL-Na may ameliorate small intestinal damage and evaluated its effects on indomethacin-induced small intestinal injuries in rats.

## MATERIALS AND METHODS

### Animals

Six-week-old male Sprague-Dawley rats (body weights of 160-200 g) were purchased from Japan SLC, Shizuoka, Japan. Animals were maintained in an air-conditioned room with controlled temperature (24 ℃ ± 2 ℃) and humidity (55% ± 15%). They were housed in steel cages with a 12 h light-dark cycle (lights on from 0700 to 1900 h). Food and water were freely available except during test periods. All animal handling procedures were conducted in accord with the guidelines for Animal Experiments of Sakai Chemical Industry.

### Induction of gastric lesions

Animals were fasted for 18 h, orally administered indomethacin (Wako, Japan) at a dose of 25 mg/kg and sacrificed after 6 h. Stomachs were removed, inflated by injecting 10 mL of 2% formalin for 10 min to fix the tissue walls, and opened along the greater curvature. Visible hemorrhagic lesions were examined, and the areas ($mm^2$) of visible lesions were calculated using Image J software.

### Induction of small intestinal lesions

Animals were not fasted, orally administered indomethacin at a dose of 10 mg/kg and sacrificed after 24 h under deep ether anesthesia. The small intestines were removed, and the organ length and wet weight were measured. Evans blue (1 mL; Sigma Aldrich Corp., St. Louis, MO, United States) was intravenously injected into the animals 30 min before sacrifice. The small intestines were opened along the anti-mesenteric attachment and examined for lesions, and the areas ($mm^2$) of visible lesions were calculated using Image J software. Blood sampling was conducted before Evans blue injection, and hematocyte numbers were determined using an automated blood cell counter (KX-21NV; Sysmex, Japan).

### Drug administration

AL-Na was obtained from Kyosei pharmaceutical (Japan), and low molecular weight AL-Na was provided by Kaigen (Japan). AL-Na and low molecular weight AL-Na were dissolved in distilled water. Rebamipide (Mucosta; Otsuka Pharmaceutics, Japan) was suspended in a 0.5% carboxymethylcellulose (CMC-Na; Wako) solution. In the small intestinal studies, AL-Na (250 and 500 mg/kg), low molecular weight AL-Na (500 mg/kg), rebamipide (100 mg/kg), and CMC-Na (250 mg/kg) were orally administered 30 min before and 6 h after treatment with indomethacin[29]. Indomethacin-treated control animals were administered distilled water at the same time.

### Assessment of myeloperoxidase activity

Myeloperoxidase (MPO) activity in the stomach and small intestine were measured. The animals were sacrificed under deep ether anesthesia, and the stomachs and small intestines were removed. After rinsing the tissues with saline, the mucosa was scraped, weighed, and homogenised in 50 mmol/L phosphate buffer containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB, pH 6.0; Sigma). Homogenised samples were frozen, thawed twice and then centrifuged at 8000 *g* for 20 min at 4 ℃. Supernatants were then assayed using a fluorometric detection kit (Assay Designs, Taiwan). Changes in fluorescence were measured using a plate reader with 545 nm excitation and 590 nm emission filters (ARVOsx; Wallac, United States).

### Determination of mucosal microvascular permeability

Microvascular permeability was evaluated in intestinal mucosa following treatment with indomethacin by measuring the amount of extravasated Evans blue dye.

After the removal of ileal tissue, the tissue was diluted with 1N KOH (0.7 mL) at 37 ℃ for 24 h. Acetone-phospholic acid was then added, and the sample was shaken. The sample was prepared after deposit filtration. The quantity of accumulated dye in the sample dilution was measured after 30 min using a spectrophotometer (JASCO; V-560, Japan) at 620 nm and was expressed as μg per 100 mg wet tissue.

### Determination of superoxide dismutase content, glutathione peroxidase activity, and catalase activity

After rinsing the ileal tissues with saline, the mucosa was scraped, weighed, and homogenised in 50 mmol/L phosphate buffer containing 5 mmol/L Tris-HCl buffer solution (pH 7.4), 5 mmol/L EDTA, and 1 mmol/L 2-mercaptoethanol. After centrifugation at 15000 g for 20 min at 4 ℃, the superoxide dismutase (SOD) content of the supernatants was determined using a superoxide dismutase ELISA kit (Northwest Life Science Specialties; NWLSS, United States). Subsequently, the glutathione peroxidase (GPx) and catalase activities were measured using a glutathione peroxidase assay kit (NWLSS) and a catalase activity assay kit (BioVision, United States). The absorbance was measured at 450, 340, and 570 nm using a plate reader (iEMS reader MF; Labsystems, Finland).

### Measurement of mucin content

Rats were anesthetised with diethyl ether, and the small intestines were excised. The luminal contents were collected by flushing with 15 mL of ice-cold PBS (pH 7.4) containing 0.02 mol/L sodium azide and the same volume of air. The contents were freeze-dried and stored for luminal mucin analysis. Total freeze-dried samples were suspended in a sodium chloride solution (0.15 mol/L) containing 0.02 mol/L sodium azide at 4 ℃. The samples were homogenised for 1 min and immediately centrifuged at 10000 *g* for 30 min to obtain the supernatant. Mucin was recovered as a 60% ethanol precipitate of the supernatant and was dissolved in 3.0 mL of distilled water for analyses using a MUC2 enzyme-linked immunosorbent assay kit (USCN life science, China). Absorption at 450 nm was measured using a plate reader (ARVOsx).

### Measurement of enterobacterial count in the intestinal mucosa

After rinsing the ileal tissues with saline, the mucosa was scraped, weighed and homogenised in 1 mL of sterile PBS per 100 mg of wet tissue. Aliquots of the homogenate were placed on blood agar and Gifu anaerobic medium (GAM) agar (Nissui, Osaka, Japan). Blood agar plates were incubated at 37 ℃ for 24 h under aerobic conditions, and GAM agar plates were incubated at 37 ℃ for 24 h under anaerobic conditions (BBL GasPack Pouch Anaerobic System, BectonDickinson, MD). Both types of plates, containing between 10 and 200 colony-forming units (CFU), were analysed and summed to determine the total number of enterobacteria, and the number of enterobacteria that were present the small intestine was expressed as log CFU/g tissue.

### Pathological studies

Stomach and ileal tissues were immediately fixed in 10% neutral buffered formalin. After fixation, the materials were dehydrated with ethanol, cleared using xylene, and embedded in paraffin. From these specimens, 3 μm paraffin sections were stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) for immunohistochemical analyses. The sections were cut, dewaxed, rehydrated, and immersed in methanol containing 0.3% (w/v) $H_2O_2$ for 30 min to inactivate endogenous peroxidases. Sections were incubated overnight at 4 ℃ with monoclonal mouse anti-proliferating cell nuclear antigen (PCNA; Dako, Denmark). After washing with PBS, the slides were incubated for 30 min with biotinylated horse anti-mouse serum (Vector, Burlingame, United States) followed by avidin-conjugated horseradish peroxidase (Vector, Burlingame, United States). The enzyme activity was detected using DAB (3,3'-diaminobenzidine).

### Evaluation of indomethacin absorption

Blood samples were collected 1 h after administration of indomethacin for evaluation of its absorption. The concentration of indomethacin in plasma was measured using high-performance liquid chromatography (HPLC). The HPLC system consisted of an AS-2055 injector, a UV-2070 detector, and a PU-2080 chromatographic pump (Nihonbunkoh, Japan). Separation was achieved on a reversed-phase column (4.6 mm × 250 mm, 3 μm, ODS, Waters, United States). The mobile phase was acetonitrile-phosphoric acid (60:40 v/v), and the flow-rate was 0.8 mL/min. The chromatogram was monitored at a wavelength of 254 nm throughout the experiments. Blood samples were centrifuged at 500 *g* for 20 min and subsequently at 1000 *g* for 20 min. The resulting plasma was mixed with 0.15 mL of acetonitrile containing 5 μg/mL of the internal standard, mefenamic acid. Denatured protein precipitates were separated using a solid-phase extraction cartridge (Waters). The extract was evaporated at 40 ℃ under $N_2$ gas, and 100 μL of the supernatant was redistilled into the mobile phase and injected into the HPLC column.

### Statistical analysis

All data are presented as the mean ± SE. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Dunnett's test or Student's unpaired *t* test. Significant differences were indicated by *P* values less than 0.05.

## RESULTS

### Effects of drugs on indomethacin-induced gastric lesions

Administration of indomethacin at 25 mg/kg caused

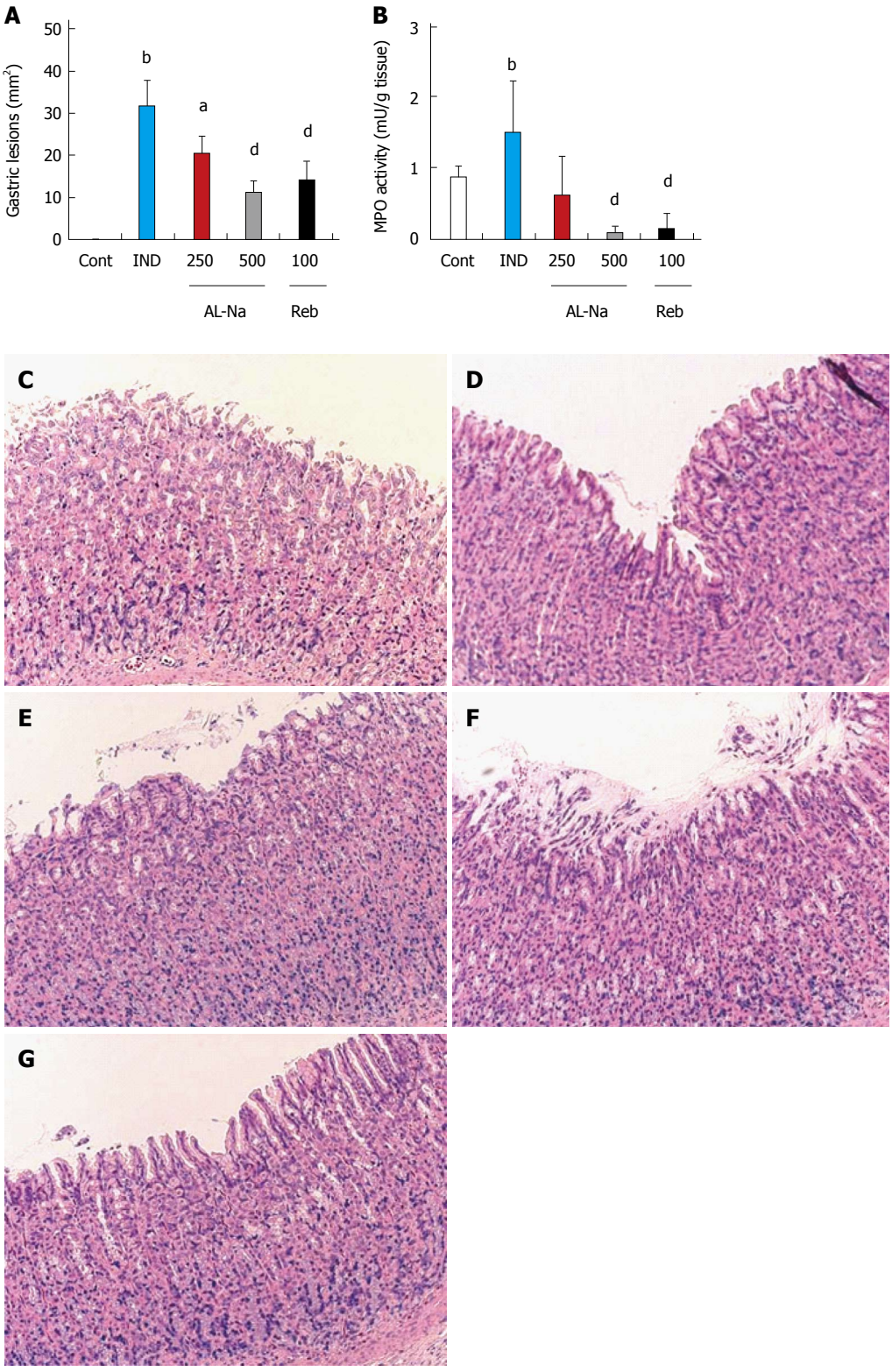


**Figure 1 Effects of drugs on indomethacin-induced gastric lesions.** Animals were given indomethacin (25 mg/kg, *po*) and killed 6 h later. AL-Na (250 and 500 mg/kg) or Reb (100 mg/kg) was given orally at 30 min before administration of indomethacin. The lesion areas (A) and myeloperoxidase (MPO) activity were measured (B). C: Hematoxylin and eosin stained microscopic observations of the rat gastric mucosa of the control (Cont) group; D: Indomethacin (IND); E: AL-Na (250 mg/kg); F: AL-Na (500 mg/kg); G: Reb (100 mg/kg). Each column and vertical bar represents the mean ± SE ($n$ = 8). Significantly different from the Cont group at $^{b}P < 0.01$ (Student's $t$-test). Significantly different from the IND group at $^{a}P < 0.05$ and $^{d}P < 0.01$, respectively (Dunnett's test). AL-Na: Sodium alginate; Reb: Rebamipide.

severe hemorrhagic lesions covering the entire glandular area of the stomach (Figure 1A). Oral treatment with AL-Na at 250 and 500 mg/kg significantly reduced the areas of indomethacin-related ulcers relative to those of indomethacin-treated control animals. Rebamipide at 100 mg/kg also reduced indomethacin-induced gastric

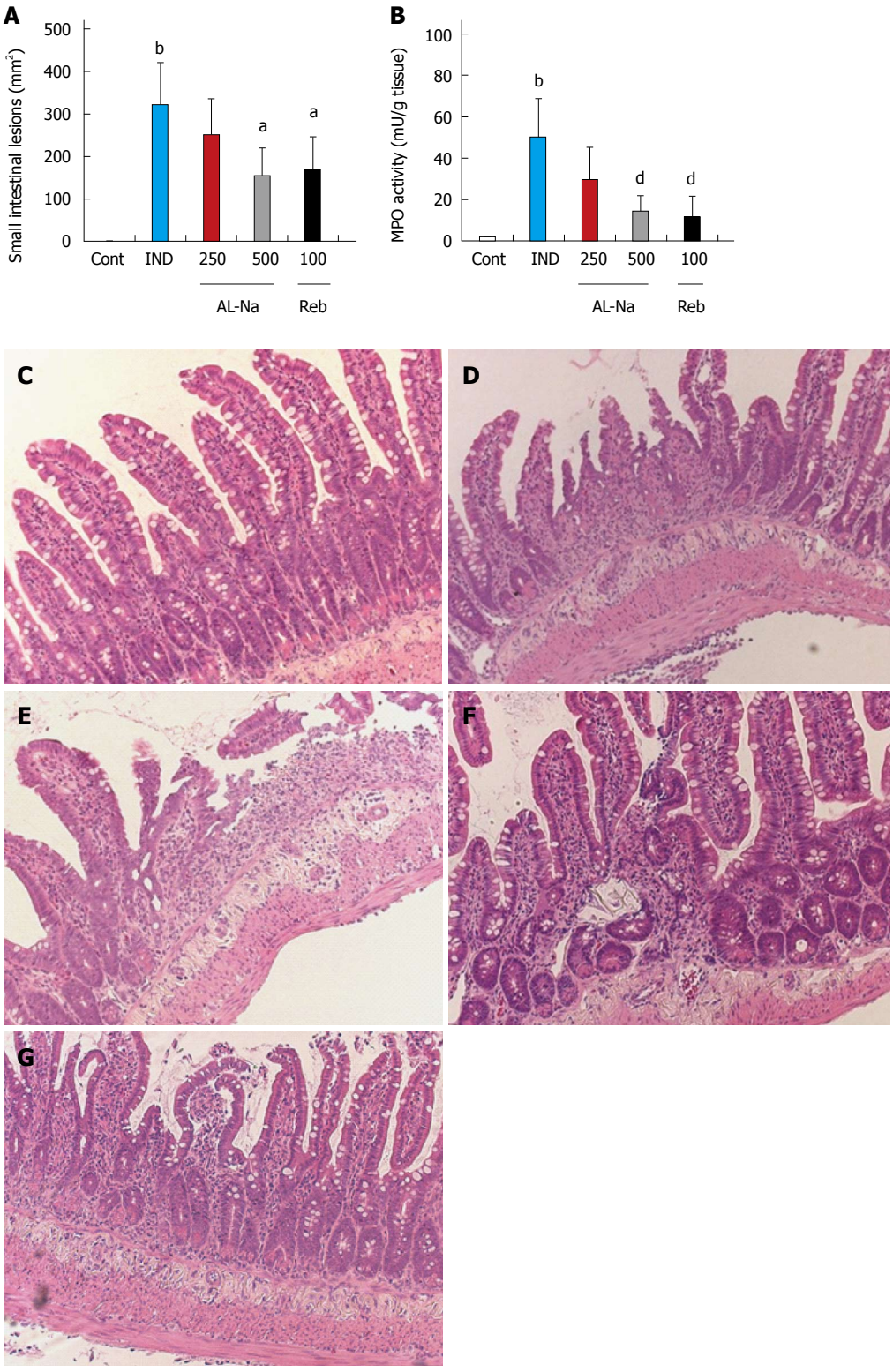


**Figure 2 Effects of drugs on indomethacin-induced small intestinal lesions.** Animals were given indomethacin (10 mg/kg, *po*) and killed 24 h later. AL-Na (250 and 500 mg/kg) or Reb (100 mg/kg) was given orally twice at 30 min before and 6 h after administration of indomethacin. (A) The lesion areas and (B) myeloperoxidase (MPO) activity were measured. C: Hematoxylin and eosin-stained microscopic observations of the rat small intestinal mucosa of the control (Cont) group; D: Indomethacin (IND); E: AL-Na (250 mg/kg); F: AL-Na (500 mg/kg); G: Reb (100 mg/kg). Each column and vertical bar represents the mean ± SE ($n$ = 8). Significantly different from the cont group at $^{b}P < 0.01$ (Student's $t$-test). Significantly different from the IND group at $^{a}P < 0.05$ and $^{d}P < 0.01$, respectively (Dunnett's test). AL-Na: Sodium alginate; Reb: Rebamipide.

lesions. Figure 1B shows the effect of AL-Na on gastric MPO induction by indomethacin. A single oral dose of 500 mg/kg AL-Na significantly inhibited indomethacin-mediated increases in MPO activity. Moreover, 100 mg/kg rebamipide also reduced indomethacin-induced MPO activity. Histological comparisons of treated and

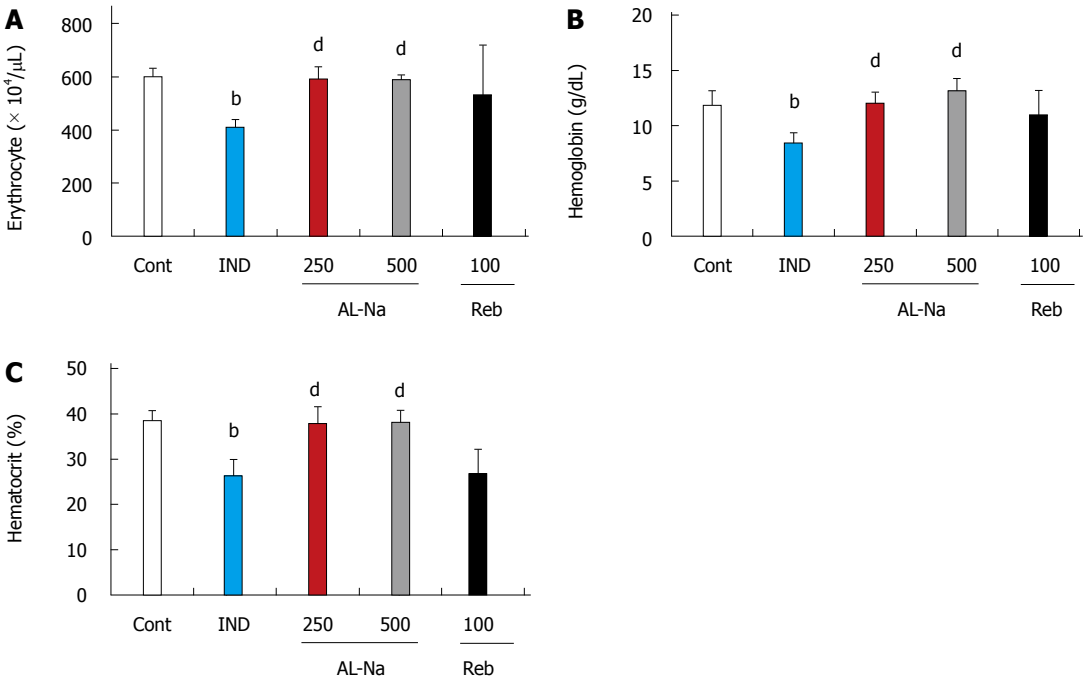


**Figure 3 Effects of drugs on indomethacin-induced anemia.** Animals were given indomethacin (10 mg/kg, *po*), and blood samples were obtained 24 h later. AL-Na (250 and 500 mg/kg) or Reb (100 mg/kg) was given orally twice at 30 min before and 6 h after administration of indomethacin. A: Erythrocyte; B: Haemoglobin; C: Haematocrit. Each column and vertical bar represents the mean ± SE ($n$ = 8). Significantly different from the control group at $^{b}P < 0.01$ (Student's $t$-test). Significantly different from the indomethacin group at $^{d}P < 0.01$ (Dunnett's test). Cont: Control; IND: Indomethacin; AL-Na: Sodium alginate; Reb: Rebamipide.

untreated tissues indicated that indomethacin caused exfoliation of gastric epithelial cells and disrupted the mucosal layer of the stomach (Figure 1D), compared with the controls (Figure 1C). Administration of AL-Na alleviated the ulceration induced by indomethacin (Figure 1E and F). Rebamipide also ameliorated indomethacin-induced gastric injury (Figure 1G).

### *Effects of drugs on indomethacin-induced small intestinal lesions*

Administration of indomethacin at 10 mg/kg caused severe hemorrhagic lesions in the small intestine, primarily the jejunum and ileum (Figure 2A). Oral treatment with AL-Na at 500 mg/kg significantly reduced the areas of indomethacin-related ulcers compared to those of the indomethacin-treated control animals. Rebamipide at 100 mg/kg also reduced indomethacin-induced small intestinal lesions. Figure 2B shows the effect of AL-Na on intestinal MPO induction by indomethacin. A single oral dose of 500 mg/kg AL-Na significantly inhibited indomethacin-mediated increases in MPO activity. Moreover, 100 mg/kg rebamipide also reduced indomethacin-induced MPO activity. Histological comparisons of treated and untreated tissues indicated that indomethacin caused an inflammatory reaction that was characterised by epithelial losses; ulcers; inflammatory infiltration into the lamina propria, submucosa, and serosa; and shortening of crypts (Figure 2D) compared with indomethacin-untreated groups (Figure 2C). The severity of these inflammatory reactions was reduced in animals treated with AL-Na (Figure 2E and F). Rebamipide also ameliorated indomethacin-induced small intestinal injury (Figure 2G).

**Table 1 Effects of drugs on loss of body weight, food intake and feces weight**

| | Change body weight (g) | Change food intake (g/rat) | Change feces weight (g/rat) |
|---|---|---|---|
| Control | 7.5 ± 2.2 | 19.7 ± 1.4 | 6.3 ± 0.7 |
| Indomethacin | -5.2 ± 5.8$^{b}$ | 8.5 ± 2.6$^{b}$ | 3.5 ± 0.6$^{b}$ |
| AL-Na (250 mg/kg) | 1.8 ± 7.6 | 15.1 ± 4.2$^{a}$ | 4.7 ± 1.2 |
| AL-Na (500 mg/kg) | 7.5 ± 2.8$^{d}$ | 19.2 ± 2.3$^{d}$ | 5.8 ± 0.8$^{d}$ |
| Reb (100 mg/kg) | -1.2 ± 4.9 | 15 ± 3.1 | 5 ± 0.7 |

Differences of weigh change 24 h after indomethacin were measured. Data are presented as the means ± SE ($n$ = 8). Significantly different from the control group at $^{b}P < 0.01$ (Student's $t$-test). Significantly different from the indomethacin group at $^{a}P < 0.05$ and $^{d}P < 0.01$ (Dunnett's test). AL-Na: Sodium alginate; Reb: Rebamipid.

### *Effects of drugs on body weight, food intake, and feces weight*

Indomethacin caused decreases in body weight, daily food intake, and feces weight. These changes were almost completely ameliorated by treatment with AL-Na (Table 1), with significant differences observed in the decreases of body weight, food intake, and feces weight between indomethacin-treated control and AL-Na-treated (500 mg/kg) animals. In contrast, rebamipide did not prevent decreases in body weight, food intake, or feces weight.

### *Effects of drugs on indomethacin-induced anemia*

Figure 3 shows the effects of AL-Na on indomethacin-

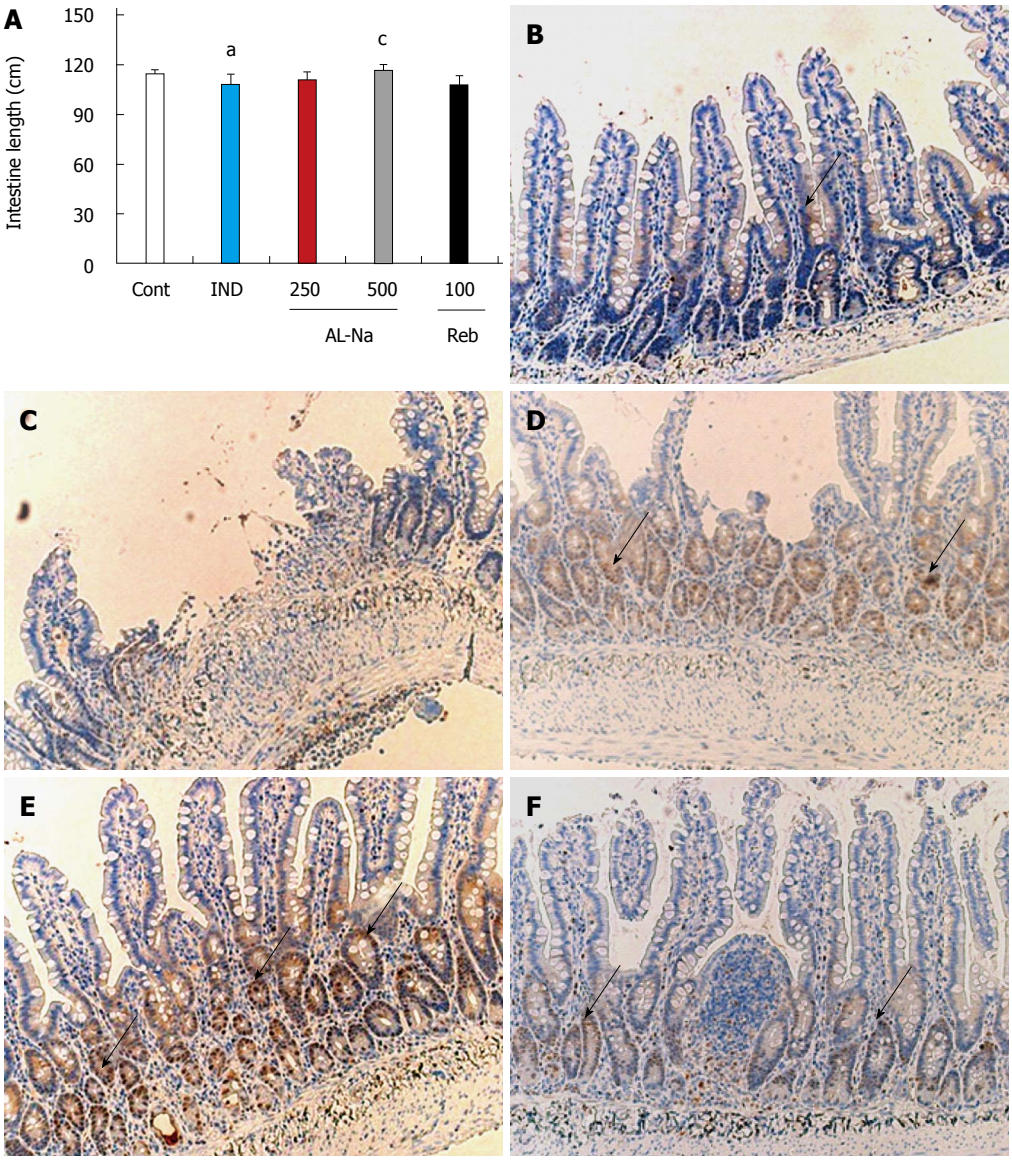


**Figure 4 Effects of drugs on indomethacin-induced atrophy.** Animals were given indomethacin (10 mg/kg, *po*) and killed 24 h later. AL-Na (250 and 500 mg/kg) or Reb (100 mg/kg) was given orally twice at 30 min before and 6 h after administration of indomethacin. A: The small intestinal length was measured; B: Microscopic observations with proliferating cell nuclear antigen (PCNA) positive cells of the rat ileal mucosa of the control (Cont) group; C: indomethacin (IND); D: AL-Na (250 mg/kg); E: AL-Na (500 mg/kg); F: Reb (100 mg/kg). Each column and vertical bar represents the mean ± SE ($n$ = 8). Significantly different from the Cont group at $^{a}P < 0.05$ (Student's $t$-test); Significantly different from the IND group at $^{c}P < 0.05$ (Dunnett's test). AL-Na: Sodium alginate; Reb: Rebamipide. The allows show PCNA positive cells.

induced anemia. The administration of 10 mg/kg indomethacin lead to decreased blood cell numbers. The number of erythrocytes, hemoglobin levels, and hematocrit were significantly reduced compared with those in untreated rats (Figure 3A-C). Oral administration of AL-Na at 250 or 500 mg/kg significantly preserved the erythrocyte numbers, hemoglobin levels, and hematocrit. In contrast, rebamipide at 100 mg/kg had no significant effect on these parameters.

### *Effects of AL-Na on indomethacin-induced atrophy of the small intestine*

Figure 4A shows the effects of AL-Na on indomethacin-induced atrophy of the small intestine. The administration of indomethacin reduced the length of the small intestines compared with those of the untreated animals. A dose of 500 mg/kg AL-Na significantly ameliorated the losses in intestine length compared with those of indomethacin-treated control rats. However, a dose of 100 mg/kg rebamipide showed no significant effect. To confirm these data, we performed immunostaining for PCNA. As shown in Figure 4B, only a few crypt cells were positive for PCNA in the animals treated with indomethacin alone, and disintegration of the crypt structures was observed (Figure 4C). In contrast, strong PCNA staining was detected in ileal crypts of animal tissues treated with AL-Na (500 mg/kg) (Figure 4D). However, rebamipide at 100 mg/kg had no effect on PCNA staining in indomethacin-treated control animals (Figure 4F).

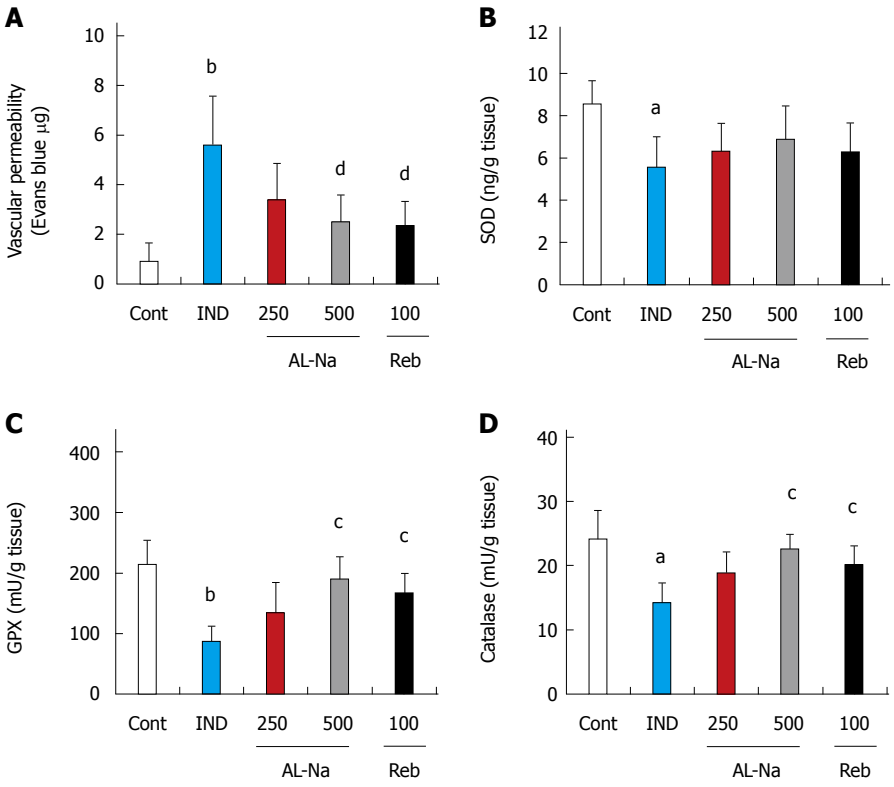


**Figure 5 Effects of drugs on indomethacin-induced vascular permeability and oxidative stress.** Animals were given indomethacin (10 mg/kg, *po*) and killed 24 h later. AL-Na (250 and 500 mg/kg) or Reb (100 mg/kg) was given orally twice at 30 min before and 6 h after administration of indomethacin. A Vascular permeability; B: Superdismdeoxidase content; C: Glutathione peroxidase activity; D: Catalase activity were measured. Each column and vertical bar represents the mean ± SE ($n$ = 8). Significantly different from the control group at $^{a}P < 0.05$ and $^{b}P < 0.01$ (Student's $t$-test); Significantly different from the indomethacin group at $^{c}P < 0.05$ and $^{d}P < 0.01$, respectively (Dunnett's test). Cont: Control; IND: Indomethacin; AL-Na: Sodium alginate; Reb: Rebamipide.

### *Effects of drugs on vascular permeability and oxidative stress in the small intestine*

Figure 5 shows the effects of AL-Na on vascular permeability and oxidative stress induced by indomethacin. Indomethacin significantly increased ileal vascular permeability (Figure 5A). Oral doses of 500 mg/kg AL-Na or 100 mg/kg rebamipide significantly inhibited indomethacin-induced vascular permeability. At a dose of 10 mg/kg, indomethacin significantly reduced the SOD, GPx, and catalase activities in ileal tissues (Figure 5B-D). At 500 mg/kg, AL-Na significantly restored the GPx and catalase activities in indomethacin-treated control rats. At 100 mg/kg, rebamipide also inhibited indomethacin-mediated decreases in the GPx and catalase activities.

### *Influence of drugs on absorption of indomethacin*

Plasma indomethacin concentrations were measured using HPLC. In plasma from indomethacin-treated control animals, indomethacin was detected at 30.7 ± 7.3 mg/mL. In animals that were treated with AL-Na at 250 mg/kg or 500 mg/kg, the indomethacin concentrations were not different from those in indomethacin-treated control animals, with 32.5 ± 7.0 mg/mL and 31.5 ± 5.8 mg/mL detected, respectively. Rebamipide treatment also had no effect, with 31.5 ± 5.7 mg/mL indomethacin detected in the plasma of these animals.

### *Effects of drugs on indomethacin-induced mucin depletion*

Figure 6A shows the effects of AL-Na on indomethacin-induced mucin depletion. Administration of 10-mg/kg indomethacin decreased the MUC2 protein levels. Oral doses of 500 mg/kg AL-Na significantly inhibited this decrease in MUC2 protein. Rebamipide at 100 mg/kg also reduced the indomethacin-induced decreases in MUC2 protein levels. Hence, we examined goblet cells, which are the major source of mucin, through the PAS staining of ileal tissues. Indomethacin-treated control animals showed depleted goblet cell numbers (Figure 6C) compared to untreated animals (Figure 6B). Treatment with AL-Na or rebamipide ameliorated this indomethacin-induced deficiency (Figure 6D-F).

### *Effects of AL-Na on changes in enterobacterial count in small intestinal mucosa*

Indomethacin caused a marked increase in the mucosal invasion of enterobacteria (Table 2). Administration of AL-Na (500 mg/kg) significantly decreased the number of enterobacteria compared with the indomethacin-treated control group.

### *Effects of low molecular AL-Na on indomethacin-induced small intestinal lesions*

Oral administration of 5% low molecular weight (7.26

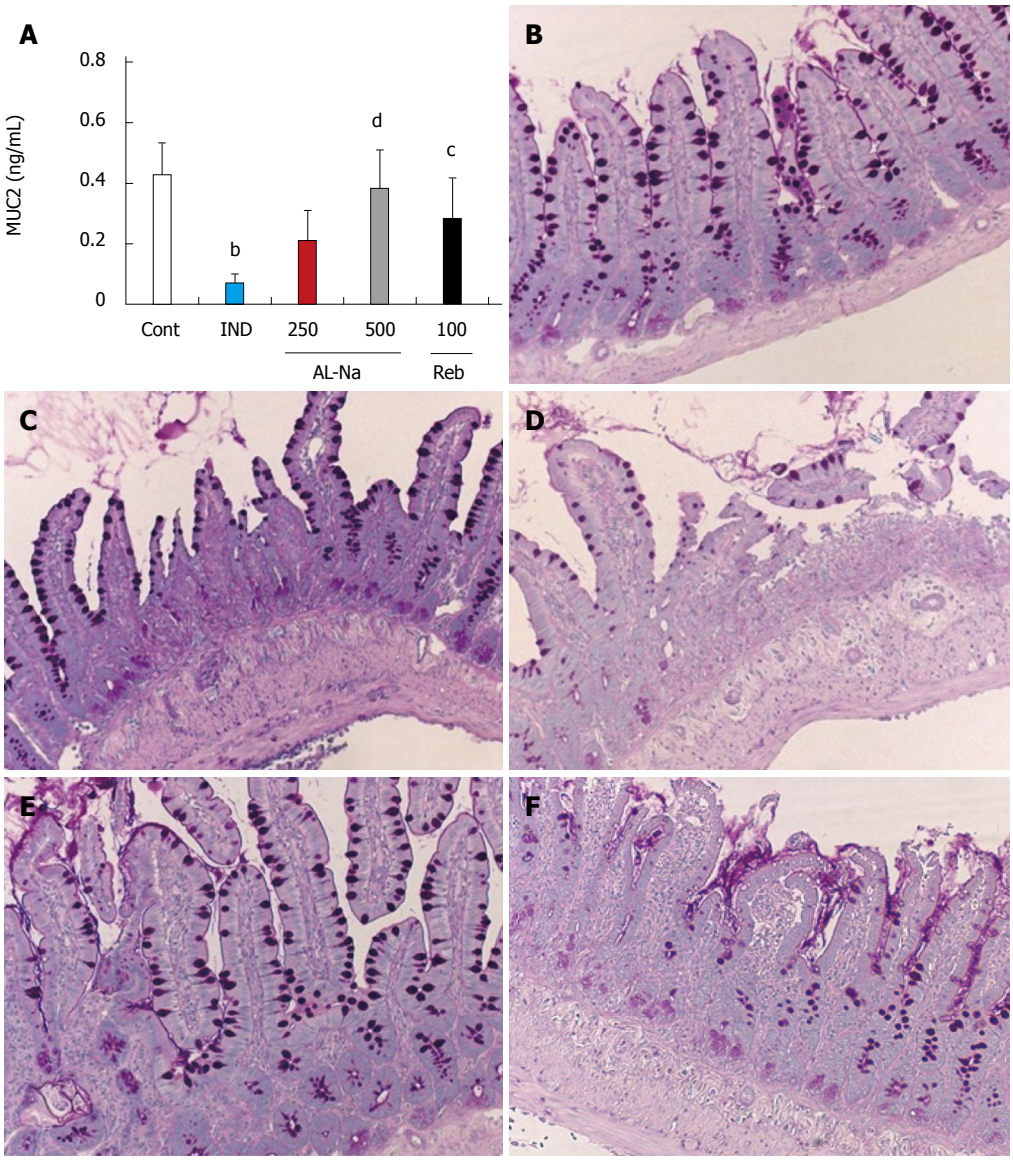


**Figure 6 Effects of drugs on indomethacin-induced mucin depletion in small intestine.** Animals were given indomethacin (10 mg/kg, *po*) and killed 24 h later. AL-Na (250 and 500 mg/kg) or Reb (100 mg/kg) was given orally twice at 30 min before and 6 h after administration of indomethacin. A: The mucin of the small intestine was measured; B: PAS-stained microscopic observations of the rat small intestinal mucosa of the control (Cont) group; C: Indomethacin (IND); D: AL-Na (250 mg/kg); E: AL-Na (500 mg/kg); F: Reb (100 mg/kg). Each column and vertical bar represents the mean ± SE ($n$ = 8). Significantly different from the Cont group at $^{b}P < 0.01$ (Student's $t$-test). Significantly different from the IND group at $^{d}P < 0.01$ and $^{c}P < 0.05$ (Dunnett's test). AL-Na: Sodium alginate; Reb: Rebamipide.

± 0.6 mPas) or original AL-Na solutions (360 ± 4.4 mPas) at a dose of 500 mg/kg significantly reduced indomethacin-induced small intestinal lesions and vascular permeability compared with those of indomethacin-treated control rats (Figure 7). However, treatments with 2.5% CMC-Na solutions, which show similar viscosity to original AL-Na (CMC-Na, 376 ± 5.6 mPas), did not affect indomethacin-induced small intestinal lesions or vascular permeability.

## DISCUSSION

Previous studies have demonstrated that AL-Na layers cover lesions, inhibit the lytic actions of pepsin and hydrochloric acid, and protect the mucosal surface of the upper gastrointestinal tract[17]. These studies also show that AL-Na enhances the production of gastric hexosamine in hydrochloric acid-induced gastric ulcers in rats[30]. Presently, we confirmed the effects of AL-Na on indomethacin-induced gastric ulcers, and additional protection from indomethacin-induced small intestinal injury, symptoms of anemia, reduction of intestinal length, increases in inflammatory response, and oxidative stress in small intestine has been observed. has been observed. Next, we tested the influence of AL-Na on indomethacin plasma concentration, and results in AL-Na had no influence. Therefore, the effect of AL-Na on indomethacin-induced gastrointestinal injury is not thought to be involved in the inhibition of indomethacin absorption. Several studies have reported intestinal bleeding and chronic anemia associated with indomethacin[2,31]. Moreover, decreased haemoglobin levels have

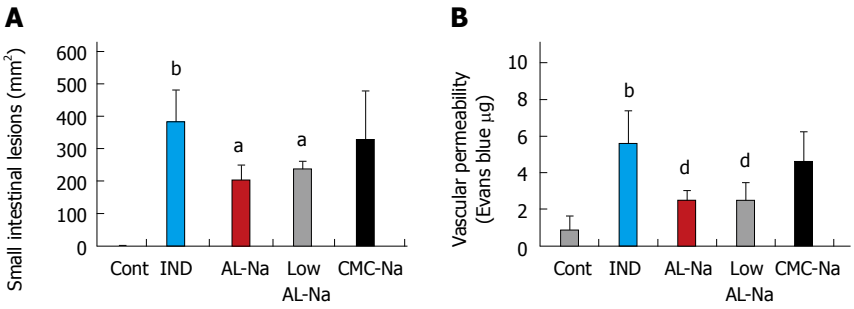


**Figure 7 Effects of low molecular sodium alginate on indomethacin-induced small intestinal lesions.** Animals were given indomethacin (10 mg/kg, *po*) and killed 24 h later. Original AL-Na (500 mg/kg), low AL-Na (500 mg/kg) or CMC-Na (250 mg/kg) was given orally twice at 30 min before and 6 h after administration of indomethacin. (A) The lesion areas were measured. (B) The vascular permeability was measured. Each column and vertical bar represents the mean ± SE ($n$ = 8). Significantly different from the control group at $^{b}P < 0.01$ (Student's $t$-test). Significantly different from the indomethacin group at $^{a}P < 0.05$ and $^{d}P < 0.01$ (Dunnett's test). Cont: Control; IND: Indomethacin; AL-Na: Sodium alginate; Low AL-Na: Low molecular sodium alginate; CMC-Na: Sodium carboxymethylcellulose.

**Table 2 Effects of sodium alginate on number of enterobacteria in ileal mucosa**

| | *n* | Total number of enterobacteria log CFU/g tissue |
|---|---|---|
| Control | 6 | 6.84 ± 0.44 |
| Indomethacin | 6 | 7.82 ± 0.42[b] |
| AL-Na (250 mg/kg) | 6 | 7.26 ± 0.53 |
| AL-Na (500 mg/kg) | 6 | 6.96 ± 0.83[a] |

Number of enterobacteria 24 h after indomethacin administration was counted. Data are presented as the means ± SE. Significantly different from the control group at $^{b}P < 0.01$ (Student's $t$-test). Significantly different from the indomethacin group at $^{a}P < 0.05$ (Dunnett's test). AL-Na: Sodium alginate.

been reported as a consequence of indomethacin treatment in rodents[32]. In the present study, AL-Na also inhibited indomethacin-induced decreases in haemoglobin levels, whereas rebamipide showed no significant effect. Daigo *et al*[19] reported that AL-Na precipitated fibrinogen and increased fibrin polymerisation. They also demonstrated enhanced aggregation of platelets following AL-Na treatment[20]. Hence, we suggest that AL-Na elicits mucoprotective effects and a hemostatic effect in the lower gastrointestinal tract in addition to protective effect on stomach.

Subsequently, we focused on the effects of AL-Na in small intestinal mucosa. The importance of mucus to the physiological defence mechanisms of the gastrointestinal tract is well documented[33]. Mucin comprises highly glycosylated large glycoproteins with protein backbone structures that are rich in serine and threonine and are linked to a wide variety of *O*-linked oligosaccharides[34]. It has been reported that indomethacin decreases the mucus content of the small intestine in rats[12]. In addition, higher magnification of the surface mucus gel layer of the small intestine has been reported during the healing process of NSAIDs-induced enteritis in rats[35,36]. Therefore, it is clear that mucin plays an important role in NSAID-induced gastrointestinal disease. Barcelo *et al*[37] reported that AL-Na induced mucin secretion in rat colon. Therefore, we hypothesised that mucin induction by AL-Na is the main mechanism involved in the healing of NSAIDs-induced enteritis. Mucin genes are broadly classified as secretory or membrane-associated. MUC2, MUC5AC, MUC5B, and MUC6 have been identified as gel-forming secretory mucin proteins. MUC2 is the major mucin produced by the goblet cells of the intestinal mucosa[38]. In stomach, AL-Na increased hexosamine levels, which are glycoprotein constituting gastric mucus[30]. Next, we examined MUC2 production in rat small intestines and showed that AL-Na inhibited indomethacin-induced MUC2 reduction in goblet cell.

Mucin plays an important role in intestinal barrier function[36] and in protection from bacterial translocation[39]. Bacterial translocation is the one of main sources of pathogenicity arising from NSAIDs-induced enteritis[7]. In these studies, the mucin inducer AL-Na affected bacterial infiltration into the small intestine, and administration of AL-Na prevented an increase in the number of enterobacteria in ileal tissue. These data suggest that AL-Na-induced MUC2 production in intestinal goblet cells defended against the infiltration of enterobacteria from small intestinal injuries caused by NSAIDs, in addition to protective effect on stomach.

Satoh *et al*[40] reported that foods containing soluble, but not insoluble, dietary fibers ameliorated the induction of intestinal lesions by indomethacin. Subsequently, they reported a strong correlation between the viscosity and the muco-protective actions of soluble dietary fibers. Hence, we also tested the effects of low molecular AL-Na and CMC-Na, which have low viscosities, on indomethacin-induced small intestinal injury. Low molecular AL-Na also prevented small intestinal injury, but CMC-Na did not. Hence, we suggest that the protective effects of AL-Na are independent of viscosity. Barcelo *et al*[37] also reported that AL-Na increased the secretion of mucin, but not cellulose, in control rat colons. They reported that glucuronic acid and galacturonic acid also increased mucin content[37] and indicated that uronic acid, which is a major constituent of AL-Na, may play an important role in mucus secretion.

In conclusion, AL-Na prevented indomethacin-induced lesions in the stomach and small intestines of rats *via*

inhibiting bacterial translocation. In addition, the results suggest that the therapeutic effects of AL-Na are independent of its viscosity. Therefore, AL-Na may be an effective treatment for NSAID-induced gut and small intestinal mucosal injury.

## ACKNOWLEDGMENTS

We would like to thank Koji Takeuchi (Professor of Kyoto Pharmaceutical University, Kyoto, Japan) and Kikuko Amagase (Assistant professor of Kyoto Pharmaceutical University) for technical support and important advice.

## COMMENTS

### Background

Non-steroidal anti-inflammatory drugs (NSAIDs) damage the stomach. Small bowel injuries from these drugs have also been reported. Patients who are on long-term NSAIDs treatment develop mucosal injuries of the small intestine, including bleeding, erosion, and ulcers. Therefore, the development of a better therapeutic agent is needed. It was reported that rebamipide, one of mucosal protective agent, suppressed NSAIDs-induced intestinal injury. But, the development of more therapeutic agent is demanded.

### Research frontiers

Sodium alginate (AL-Na) elicits a muco-protective effect by covering the surface of the gastrointestinal tract. Therefore, it may be useful for the treatment of gastric and esophageal ulcers and bleeding. In this study, the authors demonstrate that the effect of AL-Na on NSAIDs-induced enteropathy in rats.

### Innovations and breakthroughs

AL-Na is a useful in the treatment of upper gastrointestinal disease. Therefore, AL-Na may be effective in both the upper and lower gastrointestinal tracts. AL-Na is reportedly an effective treatment for experimental colitis and for radiation-induced colon damage. Therefore, the authors hypothesised that AL-Na may ameliorate small intestinal damage and evaluated its effects on indomethacin-induced small intestinal injuries in rats. This is the first study to report that the effect of AL-Na on small intestinal injury.

### Applications

The study results suggest that the AL-Na is a potentially therapeutic agenta that could be used for preventing NSAIDs-induced small intestinal enteritis *via* bacterial translocation.

### Terminology

AL-Na is a polysaccharide with homopolymeric blocks of (1-4)-linked β-D-mannuronate, and its C-5 epimer α-L-glucuronate residues are widely distributed in the cell walls of brown algae.

### Peer review

This is a good descriptive study in which the authors analyzed the preventive effect of AL-Na on enteropathy induced by NSAIDs in rats. The results are interesting and suggest that AL-Na is a potential therapeutic agent that could be used for small intestinal injury induced by NSAIDs.

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ORIGINAL ARTICLE

# Exenatide improves hepatic steatosis by enhancing lipid use in adipose tissue in nondiabetic rats

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## Abstract

**AIM:** To investigate the metabolic changes in skeletal muscle and/or adipose tissue in glucagon-like peptide-1-induced improvement of nonalcoholic fatty liver disease (NAFLD).

**METHODS:** Male Wistar rats were fed either a control diet (control group) or a high-fat diet (HFD). After 4 wk, the HFD-fed rats were subdivided into two groups; one group was injected with exenatide [HFD-Ex(+) group] and the other with saline [HFD-Ex(-) group] every day for 12 wk. The control group received saline and were fed a control diet. Changes in weight gain, energy intake, and oxygen consumption were analyzed. Glucose tolerance tests were performed after 8 wk of treatment. Histological assessments were performed in liver and adipose tissue. RNA expression levels of lipid metabolism related genes were evaluated in liver, skeletal muscle, and adipose tissue.

**RESULTS:** Exenatide attenuated weight gain [HFD-Ex(-) *vs* HFD-Ex(+)] and reduced energy intake, which was accompanied by an increase in oxygen consumption and a decrease in the respiratory exchange ratio [HFD-Ex(-) *vs* HFD-Ex(+)]. However, exenatide did not affect glucose tolerance. Exenatide reduced lipid content in the liver and adipose tissue. Exenatide did not affect the expression of lipid metabolism-related genes in the liver or skeletal muscle. In adipose tissue, exenatide significantly upregulated lipolytic genes, including hormone-sensitive lipase, carnitine palmitoyltransferase-1, long-chain acyl-CoA dehydrogenase, and acyl-CoA oxidase 1 [HFD-Ex(-) *vs* HFD-Ex(+)]. Exenatide also upregulated catalase and superoxide dismutase 2 [HFD-Ex(-) *vs* HFD-Ex(+)].

**CONCLUSION:** In addition to reducing appetite, enhanced lipid use by exenatide in adipose tissue may reduce hepatic lipid content in NAFLD, most likely by decreasing lipid influx into the liver.



**Key words:** Adipose tissue; Energy expenditure; Exenatide; Glucagon-like peptide-1; Hepatic steatosis; Lipolysis; Nonalcoholic fatty liver disease

**Core tip:** Glucagon-like peptide-1 (GLP-1) is reported to improve nonalcoholic fatty liver disease (NAFLD), mainly *via* direct action on the liver. However, organs other than the liver may also be involved in regulation of hepatic lipid contents. In this study, we found significant upregulation of lipolytic genes in adipose tissue in exenatide-treated NAFLD rats. Up-regulation

of catalase, superoxide dismutase and mitochondrial morphological regulators was observed in adipose tissue. These metabolic changes were accompanied by increased oxygen consumption and decreased respiratory exchange ratio. Taken together, enhanced lipid use by GLP-1 in adipose tissue may play an important role in the improvement of NAFLD.

Tanaka K, Masaki Y, Tanaka M, Miyazaki M, Enjoji M, Nakamuta M, Kato M, Nomura M, Inoguchi T, Kotoh K, Takayanagi R. Exenatide improves hepatic steatosis by enhancing lipid use in adipose tissue in nondiabetic rats. *World J Gastroenterol* 2014; 20(10): 2653-2663 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i10/2653.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i10.2653

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is considered a hepatic manifestation of metabolic syndrome. The significant increase in the prevalence of NAFLD in the general population indicates that NAFLD is a burgeoning problem[1]. NAFLD is a chronic liver disease that is characterized by steatosis that is histologically similar to that in alcoholic liver injury, without excessive alcoholic intake or hepatitis viral infection[2,3]. Nonalcoholic steatohepatitis, a severe stage of NAFLD, frequently progresses into liver cirrhosis and hepatocellular carcinoma[4-6]. Body weight reduction and control of complicated diabetes are essential to improve NAFLD[7,8]. However, the attempts to restrict food intake and increase physical exercise are often insufficient to treat NAFLD[9].

Glucagon-like peptide-1 (GLP-1), an incretin hormone produced by intestinal L cells, is an effective therapeutic agent for type 2 diabetes mellitus[10,11]. GLP-1 regulates plasma glucose levels by promoting insulin secretion and inhibiting glucagon secretion in a glucose-dependent manner[12,13]. Exenatide is a GLP-1 receptor agonist, sharing 53% sequence homology with GLP-1[14,15]. Exenatide has a longer half-life and enhanced potency compared with GLP-1 because it is less susceptible to degradation by dipeptidyl pepdidase-4[16].

GLP-1 may also be able to treat obesity by controlling gastrointestinal motility, which may suppress appetite and promote satiety[17,18]. GLP-1 was also reported to reduce hepatic steatosis in animal models of NAFLD[19-22]. Although the mechanism underlying this effect of GLP-1 is not completely understood, earlier studies suggested that GLP-1 had direct effects on the liver by improving hepatic insulin sensitivity[19,20] and enhancing lipid hydrolysis and oxidation[21-23]. Because the GLP-1 receptor is expressed in many organs, including the brain, heart, kidney, stomach, liver, muscle, and adipose tissue[12,24], GLP-1 may reduce hepatic lipid accumulation *via* extrahepatic pathways. In particular, skeletal muscle and adipose tissue are potential targets for GLP-1. Fatty acid influx into the liver is affected by the extent of fatty acid oxidation in skeletal muscle, as well as triglyceride storage and hydrolysis in adipose tissue. Therefore, changes in lipid metabolic activities in these tissues should reduce hepatic lipid content.

We hypothesized that GLP-1 would affect lipid metabolism in skeletal muscle and/or adipose tissue, leading to the reduction of lipid influx into the liver, resulting in the suppression of hepatic lipid accumulation. In the present study, we show that exenatide enhanced triglyceride hydrolysis and fatty acid oxidation in adipose tissue during the improvement of hepatic steatosis in a high-fat diet (HFD)-induced rat model of NAFLD. Additionally, upregulation of mitochondrial morphologic regulators was observed in adipose tissue. Exenatide increased the systemic energy expenditure and decreased the respiratory exchange ratio (RER). Collectively, the enhancing effects of exenatide (and hence GLP-1) on lipid use in adipose tissue may play a role in the improvement of hepatic steatosis in NAFLD.

## MATERIALS AND METHODS

### Animals

Four-week-old male Wistar rats weighing 80 g were purchased from Japan SLC (Hamamatsu, Japan). Rats were maintained under standard conditions with a 12-h light/dark cycle. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and were approved by the Animal Care Committee of Kyushu University. The rats were divided into two groups and fed a control diet ($n$ = 8; control group) or an HFD diet ($n$ = 16). The control diet (3.73 kcal/g) comprised 20.8% protein, 4.8% fat, and 58.2% carbohydrate. The HFD (5.06 kcal/g) comprised 18.2% protein, 62.2% fat, and 19.6% carbohydrate. Following 4 wk of feeding, HFD-fed rats were subdivided into two groups ($n$ = 8 per group) and intraperitoneally injected with either 10 μg/kg body weight exenatide [Eli Lilly, Indianapolis, IN, United States; HFD-Ex(+) group] or saline [HFD-Ex(-) group] every day for 12 wk. Rats in the control group were injected with saline and fed a control diet. Body weight was measured every 4 wk. Starting from week 6 of feeding (week 2 of exenatide/saline injection), daily food consumption in each cage (2 rats) was measured every 2 wk for 6 wk and averaged levels of energy intakes were calculated. At week 16 of feeding (week 12 of injections), rats were sacrificed after an overnight fast, and the liver, gastrocnemius muscle, and epididymal white adipose tissues were removed.

### Indirect calorimetry

At week 12 of feeding (week 8 of injections), oxygen consumption ($VO_2$, mL/kg/h) and RER were determined in the HFD-Ex(-) group and HFD-Ex(+) group ($n$ = 4 per group) using an Oxymax indirect calorimeter (Columbus Instrument, Columbus, OH, United States). After 3 d of acclimation, $VO_2$ and RER were measured

**Table 1 Primer sequences**

| Gene | Forward | Reverse |
|---|---|---|
| *SREBP1c* | GGAGCCATGGATTGCACATT | AGGAAGGCTTCCAGAGAGGA |
| *FAS* | CTAGGTGGCTTTGGCCTGGA | CGAACGTGCTTGGCTTGGTA |
| *ACC1* | GTTCTGTTGGACAACGCCTTCA | GTCGCAGAAGCAGCCCATTAC |
| *LPL* | GTGACCAGGGACATGTGACTTTG | CTTGTACTTCGTTGTGGTGGGACTA |
| *HSL* | TTGCCTACTGCTGGGCTGTC | GACACGGTGATGCAGAGGTTC |
| *ApoB* | TAGCATGCTTGCTGACATAAATGGA | ATGGAGCTGCCGGAGGTAATC |
| *CPT-1* | CTGCCAGTTCCATTAAGCCACA | CAGCTATGCAGCCTTTGACTACCA |
| *LCAD* | AAGGCCTGCTTGGCATCAAC | CAGGGCCTGTGCAATTTGAGTA |
| *ACOX1* | GGCCGCTATGATGGAAATGTG | GGGCTTCAAGTGCTTGTGGTAA |
| *Catalase* | GAACATTGCCAACCACCTGAAAG | GTAGTCAGGGTGGACGTCAGTGAA |
| *SOD2* | GACTAGGCCACAGGGCATTCA | ACTCAGAAACCCGTTTGCCTCTAC |
| *TNF* | TGGCCCAGACCCTCACACTC | CTCCTGGTATGAAGTGGCAAATC |
| *MCP1* | TCACCAGCAGCAGGTGTCCCAAAGA | ACAGAAGTGCTTGAGGTGGTTGTGG |
| *Mfn1* | CCTTGTACATCGATTCCTGGGTTC | CCTGGGCTGCATTATCTGGTG |
| *Mfn2* | TCAGCCCGAGTACACCTACAGAGA | TGAGGGCCAAATGCAAGACA |
| *Opa1* | ATGCTCGCTATCACTGCCAAC | CGTTTGCCAGTAAGCAATTTAACC |
| *Dnm1* | ATGCCTGTGGGCTAATGAACAA | GTCTCGCGATACAGCGGAAG |
| *GAPDH* | GGCACAGTCAAGGCTGAGAATG | ATGGTGGTGAAGACGCCAGTA |

SREBP1c: Sterol regulatory element-binding protein-1; FAS: Fatty acid synthase; ACC1: Acetyl-CoA carboxylase 1; LPL: Lipoprotein lipase; HSL: Hormone-sensitive lipase; ApoB: Apolipoprotein B; CPT1: Carnitine palmitoyltransferase-1; LCAD: Long-chain acyl-CoA dehydrogenase; ACOX1: Acyl-CoA oxidase-1; SOD2: Superoxide dismutase 2; TNF: Tumor necrosis factor; MCP1: Monocyte chemotactic protein-1; Mfn1: Mitofusin 1; Mfn2: Mitofusin 2; Opa1: Optic atrophy-1; Dnm1: Dynamin-1; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

every 4 min for 24 h. The rats were kept in a stable environment with a temperature of 25 ℃, 12-h light/dark cycle, and airflow of 2 L/min. RER was calculated as the ratio of the volume of $CO_2$ produced to $O_2$ consumed.

### Histological analysis

The liver and epididymal adipose tissue samples were fixed in 10% formalin and embedded in paraffin. Serial sections (5 μm thick) were cut from each block. Histological features were evaluated after staining sections with hematoxylin and eosin. The numbers of lipid droplets in liver tissue sections and the diameters of adipocytes in adipose tissue sections were determined using BIOREVO BZ9000 and BZ Ⅱ (Keyence, Osaka, Japan). The numbers of hepatic lipid droplets per unit area (/mm$^2$) and the diameters in 100 adipocytes were evaluated in five animals from each group.

### Reverse transcription-polymerase chain reaction

Total RNA was prepared from all tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and cDNA was synthesized from 1.0 μg of RNA by GeneAmp RNA polymerase chain reaction (PCR) (Applied Biosystems, Hammonton, NJ, United States) with random hexamers. Real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche, Basel, Switzerland). The reaction mixture (20 μL) contained Master SYBR Green I , 4 mmol/L $MgCl_2$, 0.5 μmol/L of the upstream and downstream PCR primers, and 2 μL of first-strand cDNA as a template. To control for variations in reactions, all PCR data were normalized against glyceraldehyde 3-phosphate dehydrogenase expression. The primer sequences used in this study are listed in Table 1.

### Glucose tolerance test

At week 12 of feeding (week 8 of injections), intraperitoneal glucose tolerance tests (IPGTTs) were performed in the HFD-Ex(-) group and HFD-Ex(+) group. After a 14-h fast, the rats were injected with glucose solution (2 g/kg body weight) and serum glucose levels were measured before (0 min) and at 15, 30, 60, 90, and 120 min after glucose injection using a portable glucometer (Lifescan, Bucks, United Kingdom).

### Immunoblotting

Adipose tissue samples (250 mg) were homogenized in 1 mL of lysis buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate). The lysates were centrifuged at 8050 *g* for 20 min. The upper lipid phase was discarded and the lower aqueous phase was re-centrifuged under the same conditions. The supernatant was collected and loaded onto Mini-Protean TGX gels (Bio-Rad, Hercules, CA, United States) and transferred onto polyvinylidene difluoride membranes. After blocking the membranes with 5% albumin, immunoblotting analyses were performed using antibodies raised against AMP-activated protein kinase (AMPK), phosphorylated AMPK (P-AMPK; Cell Signaling Technology, Beverly, MA, United States), and β-actin (Santa Cruz Biotechnology, Dallas, TX, United States).

### Statistical analysis

All results are expressed as the means ± SD. Statistical analyses were performed using JMP v. 8.01 (SAS Institute, Cary, NC, United States). The differences of means were tested by Tukey-Kramer test (among 3 groups) or Kruskal-Wallis test (between 2 groups) to identify signifi-

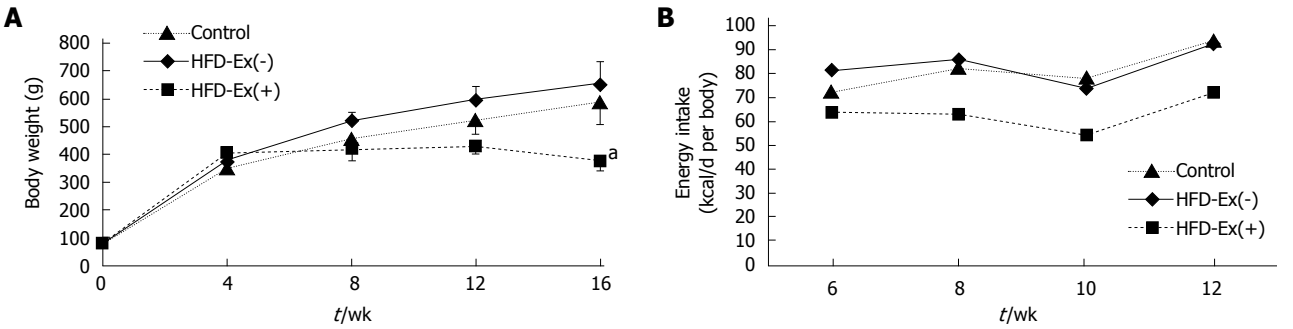


**Figure 1 Changes in body weight and energy intake.** A: Time-course of body weight. Four-week-old rats were fed a control diet (control group; $n = 8$) or a high-fat diet (HFD) ($n = 16$). After 4 wk of feeding, HFD-fed rats were subdivided into two groups ($n = 8$ per group) and intraperitoneally injected with either 10 μg/kg body weight exenatide [HFD-Ex(+) group] or saline [HFD-Ex(-) group] every day for 12 wk. Rats in the control group were injected with saline. At week 16 of feeding, the body weight was significantly lower in the HFD-Ex(+) group than in the control and HFD-Ex(-) groups. $n = 8$. $^aP < 0.05$ *vs* other groups; B: Daily energy intake during the treatment period. Energy intake was lower in the HFD-Ex(+) group than in the control or HFD-Ex(-) group.

cance. Values of $P < 0.05$ were considered statistically significant.

## RESULTS

### *Exenatide attenuated weight gain and increased oxygen consumption*

No apparent differences in weight gain were observed among the three groups after 4 wk of feeding the experimental diets. After this time, body weight gain was generally suppressed in rats treated with exenatide, whereas the body weight of rats in the control and HFD-Ex(-) groups continued to increase. At 16 wk, the body weight of rats in the HFD-Ex(+) group was significantly lower than that of the control and HFD-Ex(-) group (376 ± 36 g *vs* 587 ± 27 g and 376 ± 36 g *vs* 655 ± 81 g, respectively, $P < 0.05$) (Figure 1A). To assess the effects of exenatide on food intake, we measured energy intake (kcal/day per body) between weeks 6 and 12 of the feeding protocol. Energy intake was lower in the HFD-Ex(+) group than in the other groups throughout this time (Figure 1B). Using indirect calorimetry, we determined systemic energy consumption and RER in the HFD-Ex(-) and HFD-Ex(+) groups at week 12 of feeding. Indirect calorimetry revealed that oxygen consumption was significantly greater in the HFD-Ex(+) group than in the HFD-Ex(-) group (1269 ± 67 mL/kg/h *vs* 1114 ± 97 mL/kg/h, $P < 0.05$), and this increase was predominant during the dark cycle (Figure 2A). RER was significantly lower in the HFD-Ex(+) group than in the HFD-Ex(-) group (0.748 ± 0.02 *vs* 0.791 ± 0.01, $P < 0.05$) (Figure 2B). These findings indicate that exenatide enhanced systemic energy consumption by increasing lipid oxidation.

### *Exenatide reduced lipid accumulation in the liver and adipose tissue*

Lipid accumulation in the liver and epididymal white adipose tissue was histologically analyzed at week 16 of feeding. Although marked accumulation of lipid droplets was observed in the livers of the HFD-Ex(-) group, the number of hepatic lipid droplets was significantly decreased in the HFD-Ex(+) group compared with the HFD-Ex(-) group (Figure 3A, C). In adipose tissue, the adipocytes were frequently enlarged in the HFD-Ex(-) group, reflecting lipid accumulation. However, enlarged adipocytes were not observed in the HFD-Ex(+) or control groups (Figure 3B). Furthermore, the mean diameter of adipocytes in the HFD-Ex(+) group was similar to that in the control group and was significantly smaller in both groups than in the HFD-Ex(-) group (Figure 3D).

### *Effects of exenatide on the expressions of genes involved in lipid metabolism in the liver and skeletal muscle*

Because exenatide increased oxygen consumption, decreased RER, and decreased lipid accumulation in the liver and adipose tissue, we hypothesized that exenatide altered lipid metabolic activities, including triglyceride hydrolysis and lipid oxidation. To confirm this hypothesis, we determined the expression of lipid metabolism-associated genes in the liver, skeletal muscle, and adipose tissue. In liver, we found that the expression levels of sterol regulatory element-binding protein-1c (SREBP1c), fatty acid synthase (FAS), acetyl-CoA carboxylase-1 (ACC1), hormone-sensitive lipase (HSL), and apolipoprotein B (ApoB) were not significantly different among the three groups [HFD-Ex(-) *vs* HFD-Ex(+); 2.20 ± 1.29 *vs* 0.91 ± 0.57, 1.37 ± 0.51 *vs* 1.15 ± 0.68, 1.03 ± 0.29 *vs* 1.03 ± 0.29, 1.61 ± 0.69 *vs* 1.27 ± 0.21, and 0.99 ± 0.29 *vs* 0.89 ± 0.15, respectively, $P > 0.05$] (Figure 4A). Additionally, exenatide did not affect the expression of carnitine palmitoyltransferase-1 (CPT1), long-chain acyl-CoA dehydrogenase (LCAD), or acyl-CoA oxidase 1 (ACOX1) [HFD-Ex(-) *vs* HFD-Ex(+); 1.06 ± 0.50 *vs* 0.95 ± 0.39, 1.11 ± 0.41 *vs* 0.83 ± 0.21, and 0.85 ± 0.14 *vs* 0.74 ± 0.25, respectively, $P > 0.05$]. In skeletal muscle, the expression of lipoprotein lipase (LPL) was significantly increased in the HFD-Ex(+) group compared with the control group [control *vs* HFD-Ex(+); 1 ± 0.60 *vs* 5.48 ± 4.47, $P < 0.05$] but not compared with the HFD-Ex(-) group [HFD-Ex(-) *vs* HFD-Ex(+); 3.24 ± 2.19 *vs* 5.48 ± 4.47, $P > 0.05$]. As in the liver, exenatide did not affect the

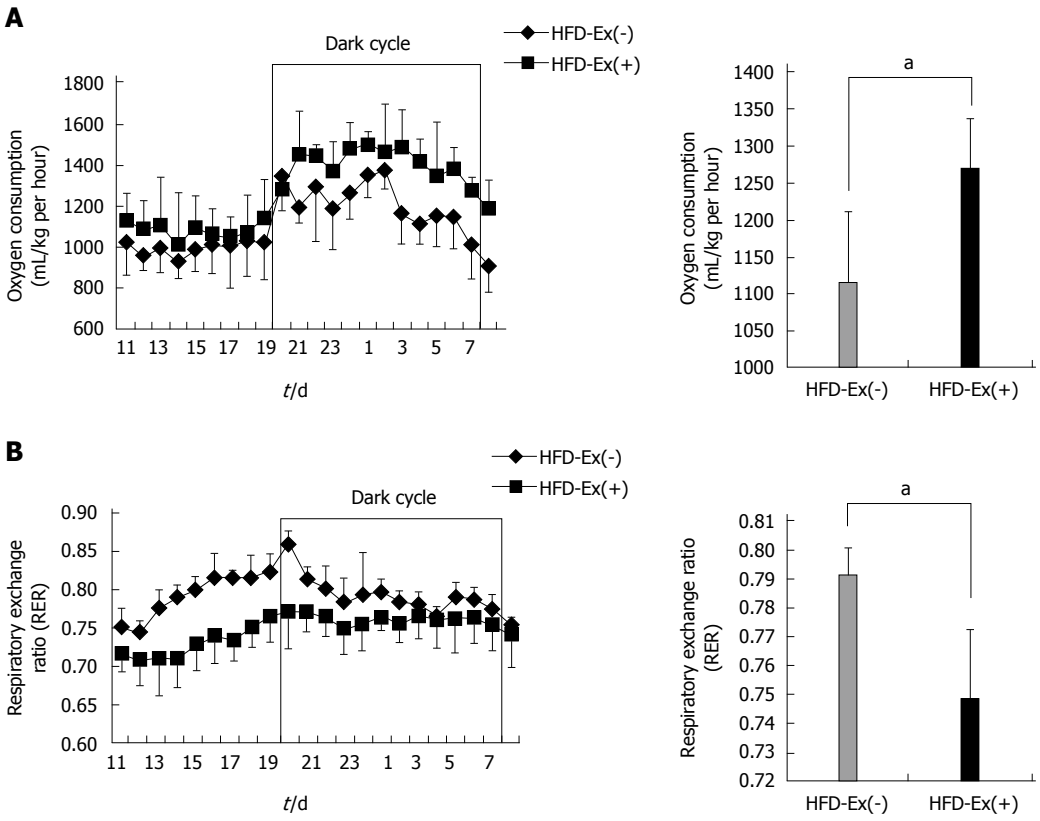


**Figure 2 Oxygen consumption and respiratory exchange ratio evaluated by indirect calorimetry in the high-fat diet-Ex(+) and high-fat diet-Ex(-) groups at week 12 of feeding.** A: Oxygen consumption was significantly greater in the high-fat diet (HFD)-Ex(+) group than in the HFD-Ex(-) group, particularly during the dark cycle; B: Respiratory exchange ratio (RER) was significantly lower in the HFD-Ex(+) group than in the HFD-Ex(-) group. $n = 4$. $^aP < 0.05$ between groups.

expression of HSL, CPT1, LCAD, or ACOX1 in skeletal muscle [HFD-Ex(-) *vs* HFD-Ex(+); 1.44 ± 0.65 *vs* 1.87 ± 0.88, 1.26 ± 0.51 *vs* 1.53 ± 0.63, 2.15 ± 0.78 *vs* 1.88 ± 0.77, and 1.40 ± 0.33 *vs* 1.32 ± 0.61, respectively, $P > 0.05$] (Figure 4B). These results imply that exenatide improves hepatic steatosis without affecting lipid metabolism in the liver or skeletal muscle, except for a potential increase in triglyceride hydrolysis in skeletal muscle.

### *Exenatide upregulated genes involved in triglyceride hydrolysis and fatty acid oxidation in adipose tissue*

In adipose tissue, exenatide significantly increased the expression of HSL in the HFD-Ex(+) group compared with the control and HFD-Ex(-) groups [HFD-Ex(-) *vs* HFD-Ex(+); 0.98 ± 0.37 *vs* 1.61 ± 0.42, $P < 0.05$] (Figure 5A). The expression of LPL was also increased in the HFD-Ex(+) group, albeit not significantly. In terms of genes involved in mitochondrial β oxidation of fatty acids, the expression levels of CPT1, LCAD, and ACOX1 were significantly increased in the HFD-Ex(+) group compared with the control and HFD-Ex(-) groups [HFD-Ex(-) *vs* HFD-Ex(+); 1.04 ± 0.27 *vs* 1.88 ± 0.97, 1.26 ± 0.23 *vs* 2.52 ± 1.00, and 1.58 ± 0.45 *vs* 2.41 ± 0.85, respectively, $P < 0.05$], suggesting that exenatide enhanced lipid oxidation in adipose tissue.

Enhanced lipid oxidation results in the accumulation of intracellular reactive oxygen species (ROS), which induces the cellular response to eliminate this harmful by-product[25-27]. Therefore, we determined the adipose tissue expression levels of catalase and superoxide dismutase (SOD)2 and found that they were significantly greater in the HFD-Ex(+) group than in the control and HFD-Ex(-) groups [HFD-Ex(-) *vs* HFD-Ex(+); 1.12 ± 0.29 *vs* 2.37 ± 0.66 and 0.99 ± 0.21 *vs* 1.49 ± 0.23, respectively, $P < 0.05$] (Figure 5B).

Because macrophage infiltration into adipose tissue plays an important role in the development of insulin resistance[28-30], we determined the expression levels of tumor necrosis factor and monocyte chemotactic protein 1. However, the expression levels of these genes were not significantly different among the three groups [HFD-Ex(-) *vs* HFD-Ex(+); 1.01 ± 0.40 *vs* 1.27 ± 0.30 and 0.90 ± 0.41 *vs* 0.76 ± 0.49, respectively, $P > 0.05$], which suggests that the metabolic changes in adipose tissues induced by exenatide did not involve macrophage activation (Figure 5B).

### *Effects of exenatide on mitochondrial morphologic regulators in adipose tissue*

In response to changes in the nutritional environment, mitochondria can change their morphology through two coordinated processes, fusion and fission, which are transcriptionally regulated by a group of genes[31,32]. In this group, mitofusin-1 (Mfn1) and mitofusin-2 (Mfn2) regulate mitochondrial fusion of the outer membrane and are believed to play a role in intracellular lipid con-

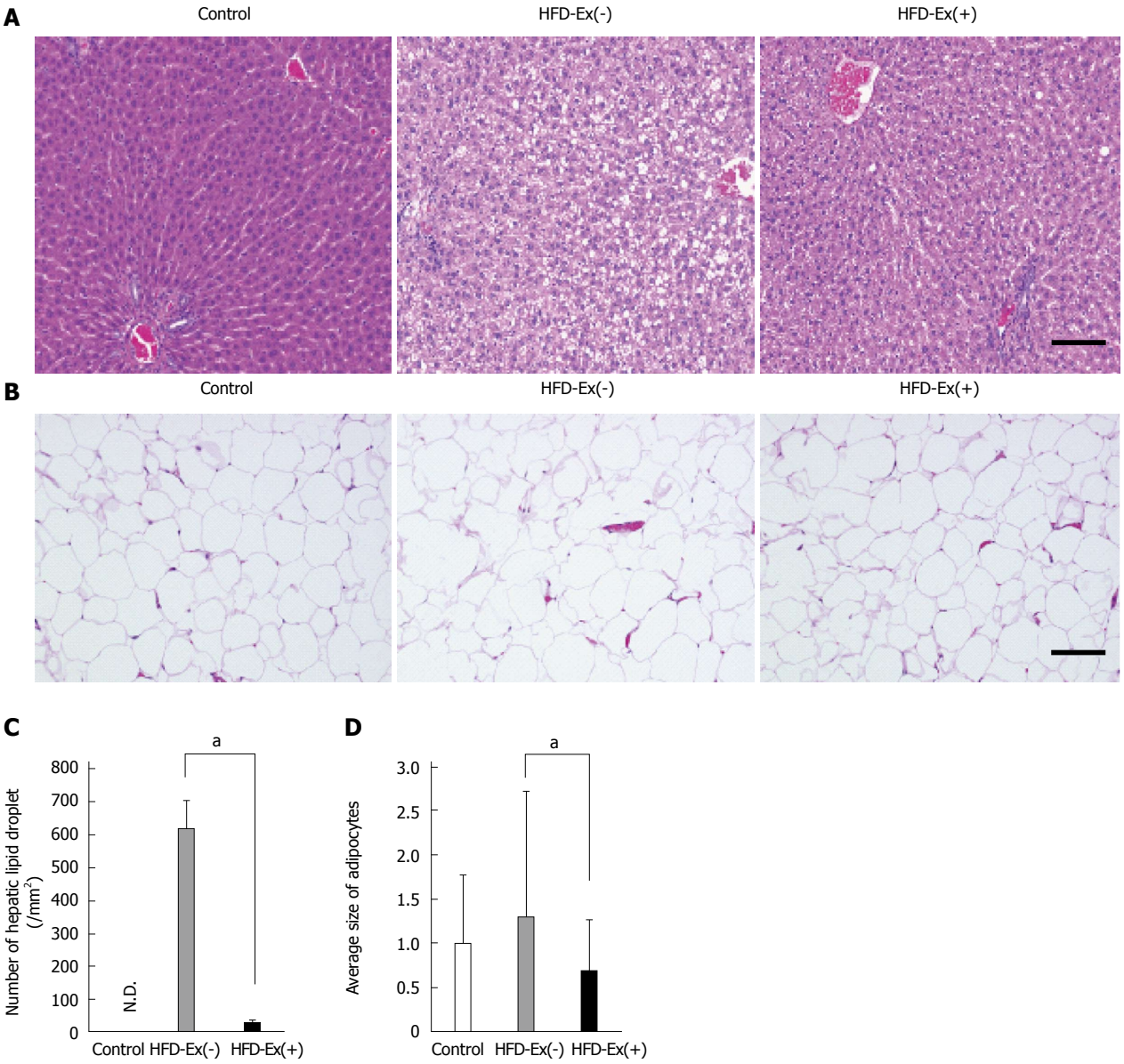


**Figure 3 Histological evaluation of lipid accumulation in the liver and adipose tissue.** A: Numerous hepatocytes containing lipid droplets were observed in the high-fat diet (HFD)-Ex(-) group, whereas scant lipid-containing hepatocytes were found in the HFD-Ex(+) group; B: In epididymal white adipose tissue, there were abundant enlarged adipocytes in the HFD-Ex(-) group but not in the HFD-Ex(+) group; C: The number of hepatic lipid droplets was significantly decreased in the HFD-Ex(+) group compared with the HFD-Ex(-) group; D: The mean diameter of adipocytes in the HFD-Ex(+) group was significantly smaller than that in the HFD-Ex(-) group and was similar to that in the control group. The fold changes were calculated as the ratio of the average size of adipocytes in the HFD-Ex(+) or HFD-Ex(-) group to that in the control group. $n = 5$, $^{a}P < 0.05$ between groups. Scale bar = 100 μm. ND: Not detected.

sumption[33]. In addition, optic atrophy-1 (Opa1) regulates the fusion of the inner membrane while dynamin-1 (Dnm1) regulates mitochondrial fission and is involved in intracellular lipid accumulation[33]. Therefore, to determine whether the induction of lipid oxidation in adipose tissue is accompanied by changes in mitochondrial morphologic regulation, we determined the expression levels of these regulators. Notably, the expression levels of Mfn1, Mfn2, and Opa1 were significantly greater in the HFD-Ex(+) group than in the control and HFD-Ex(-) group [HFD-Ex(-) *vs* HFD-Ex(+); 1.13 ± 0.17 *vs* 2.08 ± 0.40, 0.99 ± 0.28 *vs* 1.76 ± 0.50, and 1.08 ± 0.19 *vs* 1.76 ± 0.30, respectively, $P < 0.05$] (Figure 6). Additionally, the expression of Dnm1 was significantly greater in the HFD-Ex(+) group than in the control group [control *vs* HFD-Ex(+); 1 ± 0.50 *vs* 1.58 ± 0.27, $P < 0.05$], but was not significantly greater than that in the HFD-Ex(-) group [HFD-Ex(-) *vs* HFD-Ex(+); 1.28 ± 0.23 *vs* 1.58 ± 0.27, $P > 0.05$]. These findings indicate that exenatide not only induces lipid consumption or accumulation, but also that it might regulate mitochondrial reorganization of adipose tissue, most likely reflecting increased use of intracellular lipid.

### *Exenatide had limited effects on glucose tolerance*

This NAFLD model was based on nondiabetic, wild-type rats to minimize the effects of exenatide on diabetes control. However, improvement in hepatic lipid accu-

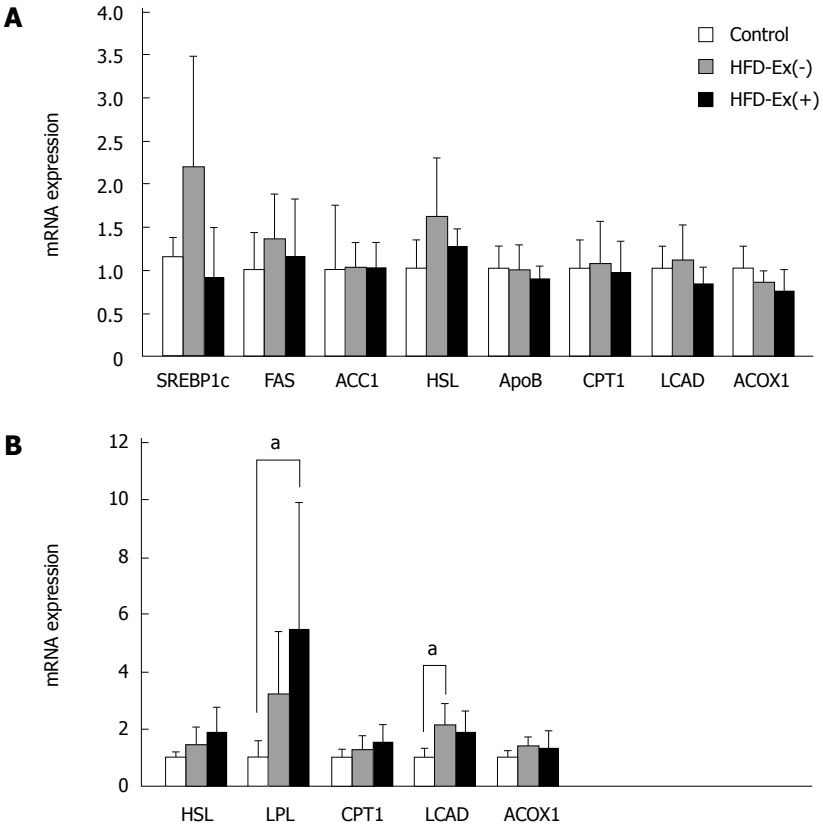


**Figure 4 Effects of glucagon-like peptide-1 on the expression levels of genes associated with lipid metabolism in the liver and skeletal muscle.** A: In the liver, there were no significant differences in the expression levels of sterol regulatory element-binding protein-1c (SREBP1c), fatty acid synthase (FAS), acetyl-CoA carboxylase-1 (ACC1), hormone-sensitive lipase (HSL), and apolipoprotein B (ApoB), carnitine palmitoyltransferase-1 (CPT1), long-chain acyl-CoA dehydrogenase (LCAD), or acyl-CoA oxidase 1 (ACOX1) among the three groups; B: In skeletal muscle, the expression of LPL was significantly greater in the high-fat diet (HFD)-Ex(+) group than in the control group. There were no significant differences in the expression levels of HSL, LPL, CPT1, LCAD, or ACOX1 between the HFD-Ex(-) and HFD-Ex(+) group. The fold changes were calculated as the ratio of the expression level in the HFD-Ex(+) or HFD-Ex(-) group to that in the control group. $n = 8$, $^{a}P < 0.05$ between groups.

mulation might be due to an improvement in glucose intolerance, which occasionally develops in obese animals. Thus, we performed IPGTTs in rats in the HFD-Ex(-) and HFD-Ex(+) groups. Interestingly, fasting plasma glucose levels were slightly higher in the HFD-Ex(-) group than in the HFD-Ex(+) group, but no significant differences were observed at 15, 30, 60, 90, or 120 min after glucose injection (Figure 7). The nondiabetic profiles and the similar responses in both groups suggest that the effects of exenatide on lipid metabolism are unlikely to be due to improvements in glucose intolerance.

### *Adipose tissue AMPK is not activated by exenatide*

Following an increase in intracellular AMP, AMPK plays an essential role in the consumption of intracellular lipid by suppressing fatty acid synthesis and stimulating fatty acid oxidation[34]. To determine whether AMPK activation was involved in the effects of exenatide, we determined the protein expression of total AMPK and P-AMPK in adipose tissue. As shown in Figure 8, there were no obvious differences in AMPK or P-AMPK levels among the three groups.

## DISCUSSION

The mechanism by which GLP-1 and its analogs improve hepatic steatosis is still not fully understood, although changes in hepatic lipid metabolism are thought to be involved in these effects[20-23]. Because hepatic lipid content is determined by intrahepatic lipogenesis and lipolysis, as well as the extent of fatty acid influx into the liver, changes in lipid use in skeletal muscle and adipose tissue may contribute to hepatic lipid metabolism. Thus, we investigated the effects of exenatide, a GLP-1 receptor agonist, on lipid metabolism in the liver, skeletal muscle, and adipose tissue. To minimize the effects of exenatide on glycemic control in the diabetic state, we used a nondiabetic, HFD-induced rat NAFLD model. Exenatide reduced lipid accumulation in the liver and in adipose tissue and decreased the size of adipocytes. The reduction of body weight gain by exenatide was accompanied by a significant reduction in food intake. Using indirect calorimetry, we showed that exenatide increased oxygen consumption and reduced the RER. These findings suggest that reduced food intake and increased

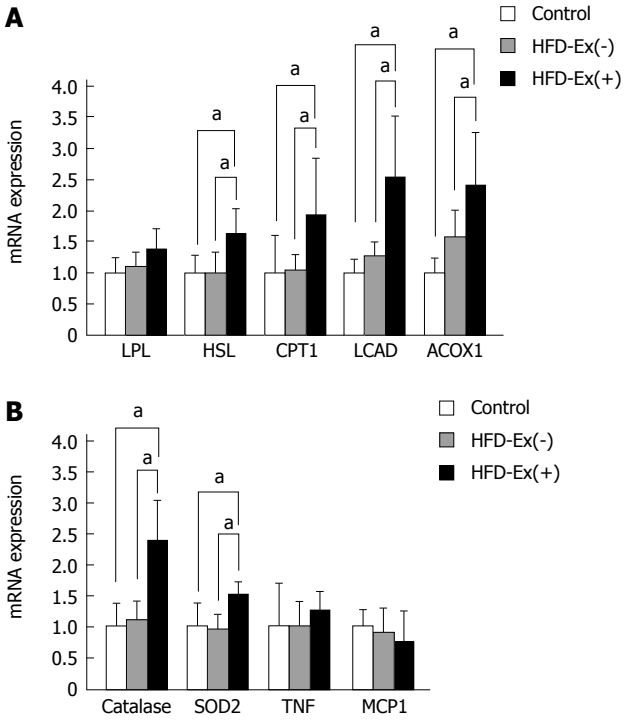


**Figure 5 Effects of exenatide on the expression levels of genes associated with lipid metabolism, reactive oxygen species elimination, and macrophage activation in adipose tissue.** A: The expression levels of hormone-sensitive lipase (HSL), carnitine palmitoyltransferase-1 (CPT1), long-chain acyl-CoA dehydrogenase (LCAD), and acyl-CoA oxidase 1 (ACOX1) were significantly greater in the high-fat diet (HFD)-Ex(+) group than in the control and HFD-Ex(-) groups; B: The expression levels of catalase and superoxide dismutase (SOD)2 were significantly greater in the HFD-Ex(+) group than in the control and HFD-Ex(-) groups. There were no significant differences in tumor necrosis factor (TNF) or monocyte chemotactic protein 1 (MCP1) expression levels among the three groups. The fold changes were calculated as the ratio of the expression level in the HFD-Ex(+) or HFD-Ex(-) group to that in the control group. $n = 8$, $^{a}P < 0.05$ between groups.

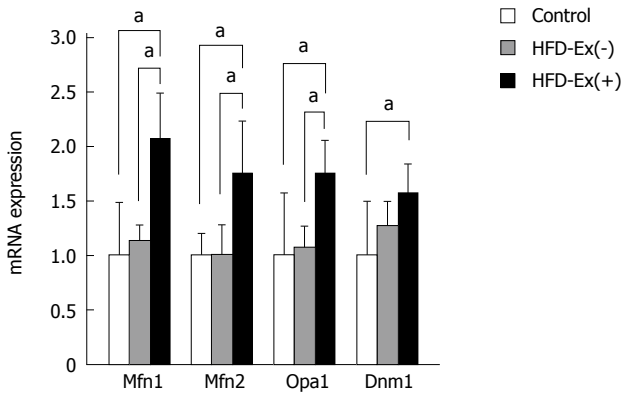


**Figure 6 Effects of exenatide on the expression levels of mitochondrial morphologic regulators in adipose tissue.** Genes involved in mitochondrial fusion [mitofusin-1 (Mfn1) and mitofusin-2 (Mfn2) and optic atrophy-1 (Opa1)] were significantly greater in the high-fat diet (HFD)-Ex(+) group than in the control and HFD-Ex(-) groups. The expression of dynamin-1 (Dnm1), which is involved in mitochondrial fission, was not significantly different between the HFD-Ex(+) and HFD-Ex(-) groups. The fold changes were calculated as the ratio of the expression level in the HFD-Ex(+) or HFD-Ex(-) group to that in the control group. $n = 8$, $^{a}P < 0.05$ between groups.

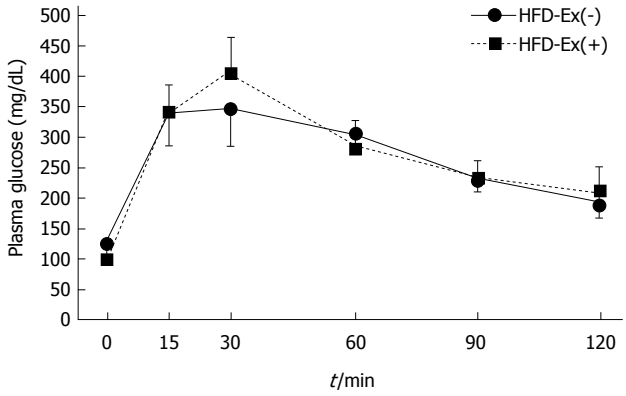


**Figure 7 Effect of exenatide on glucose tolerance.** Intraperitoneal glucose tolerance tests (IPGTTs) were performed in the high-fat diet (HFD)-Ex(+) and HFD-Ex(-) groups at week 12 of feeding. Fasting plasma glucose levels were slightly lower in the HFD-Ex(+) group than in the HFD-Ex(-) group, but no significant differences were observed at the other times during the IPGTTs.

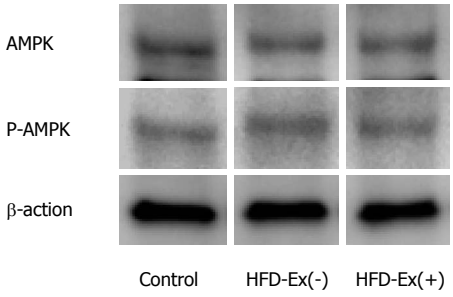


**Figure 8 Effects of exenatide on AMP-activated protein kinase activation in adipose tissue.** Immunoblotting for total AMP-activated protein kinase (AMPK), phosphorylated AMPK (P-AMPK), and β-actin were performed. The protein levels of total AMPK and P-AMPK were not significantly different among the three groups. HFD: High-fat diet.

energy consumption, most likely through increased lipid use, contribute to the exenatide-induced reduction in systemic lipid accumulation. We then determined the expression levels of lipid metabolism-related genes in the liver, skeletal muscle, and adipose tissue. Surprisingly, the hepatic expression levels of lipogenic genes (*SREBP1c*, *FAS*, and *ACC1*) and lipolytic genes (*HSL*, *CPT1*, *LCAD*, and *ACOX1*) were unaffected by exenatide. The expression of ApoB was also unaffected. These results are inconsistent with those of previous reports, which revealed that GLP-1 and its analogs directly modulate hepatic lipid metabolism[20,23,35,36]. Because these findings were mainly observed in diabetic animals or in cultured hepatocytes, we speculated that organs other than the liver might be more sensitive to GLP-1 or its analogs in the nondiabetic state, resulting in the absence of a hepatic response. In skeletal muscle, exenatide did not affect the expression levels of HSL, CPT1, LCAD, or ACOX1, but LPL expression was significantly higher in the HFD-Ex(+) group compared with the control, but not compared with the HFD-Ex(-) group, which suggests that lipid consumption is not increased in skeletal

muscle. However, lipolytic genes were upregulated by exenatide in adipose tissue. Notably, the expression levels of HSL, CPT1, LCAD, and ACOX1 were significantly greater in the HFD-Ex(+) group than in the control and HFD-Ex(-) groups. To evaluate whether these changes were associated with changes in nutrient oxidation, we analyzed the expression levels of catalase and SOD2, which are responsible for eliminating ROS produced during oxidative phosphorylation[37,38]. We showed that exenatide significantly upregulated the expression of both enzymes. Therefore, exenatide seemed to promote nutrient oxidation, especially of lipid, in adipose tissue. To analyze the effects of exenatide on lipid use, we determined the expression levels of genes regulating mitochondrial morphology, which responds to changes in the nutritional environment[33,39,40]. In particular, intracellular lipid content is greatly affected by the correlation between mitochondrial fusion and fission[31,32]. In this study, we showed that exenatide significantly upregulated the expression levels of Mfn1, Mfn2, and Opa1, which regulate mitochondrial fusion and promote the consumption of intracellular lipid[31,32]. Taken together, our observations suggest that exenatide enhanced lipid use in adipose tissue, which contributed to the improvement in hepatic steatosis, most likely by reducing lipid influx into the liver.

The mechanisms by which GLP-1 modulates lipid use in adipose tissue are largely unknown. The GLP-1 receptor has been detected in 3T3-L1 adipocytes and in human adipose tissue[24,41,42], and GLP-1 was reported to stimulate lipolysis in a receptor-dependent manner[42]. These findings suggest that exenatide enhances lipid use by signaling *via* the GLP-1 receptor in adipocytes. Another mechanism may involve activation of the sympathetic nervous system. It was reported that treatment with a dipeptidyl peptidase 4 inhibitor increased lipolysis in adipose tissues, and this was associated with elevated plasma norepinephrine levels[43]. Furthermore, intracerebroventricular infusion of GLP-1 decreased lipid storage in white adipose tissue in a manner that was partially mediated *via* sympathetic nerve activation[44]. These findings are consistent with our observation that exenatide enhanced adipose tissue expression of HSL, which is activated by the sympathetic nervous system *via* the cAMP-dependent pathway[45,46]. Not only white adipose tissue, but also brown adipose tissue might be involved in the actions of GLP-1 observed in this study. Recently, Lockie *et al*[47] reported that intracerebroventricular injection of GLP-1 induced thermogenesis in brown adipose tissue, accompanied with increased activity of innervated sympathetic fibers. Taken together, we hypothesize that GLP-1 enhances lipid utility in both the adipose tissues, lipolysis in white adipose tissue and thermogenesis in brown adipose tissue, leading to increased energy consumption, resulting in the improvement of hepatic steatosis. However, the precise roles of these adipose tissues need further investigation.

In conclusion, this study showed that reduced food intake and enhanced lipid use by exenatide in adipose tissue contributed to an improvement in hepatic steatosis in a rat model of HFD-induced NAFLD. The mechanism by which exenatide (and therefore GLP-1) modulates lipid metabolism in adipose tissue should be investigated further.

## COMMENTS

### Background

Nonalcoholic fatty liver disease (NAFLD) is considered a hepatic manifestation of metabolic syndrome. Recently, with the prevalence of metabolic syndrome, NAFLD-patients are increasing. If untreated, NAFLD or nonalcoholic steatohepatitis (NASH), a severe stage of NAFLD, frequently progresses into liver cirrhosis and hepatocellular carcinoma. Body weight reduction and control of complicated diabetes are essential to improve NAFLD. However, attempts to restrict food intake and increase physical exercise are often insufficient to treat NAFLD.

### Research frontiers

Glucagon-like peptide-1 (GLP-1) was reported to reduce hepatic steatosis in animal models of NAFLD. In addition to suppressing appetite, direct action of GLP-1 on the liver is reported to enhance hepatic lipolysis to prevent lipid accumulation. Because GLP-1 receptor distributes widely in various tissues, authors supposed that organs other than the liver might also be involved in the regulation of hepatic lipid contents. In this study using a rat model of NAFLD, authors evaluated the changes in lipid metabolism induced by GLP-1 treatment in liver, skeletal muscle, and adipose tissue.

### Innovations and breakthrough

In the high-fat diet (HFD)-induced NAFLD model, GLP-1 treatment reduced lipid accumulations in liver and adipose tissues. Authors found that increased expressions of genes were involved in lipolysis and lipid oxidation in adipose tissue, but not in the liver or skeletal muscle. In adipose tissue, GLP-1 significantly upregulated catalase, superoxide dismutase 2, and mitochondrial morphological regulators. Because the improvement of hepatic steatosis by GLP-1 was accompanied with increased energy expenditure and decreased respiratory exchange ratio, enhanced lipid utility by GLP-1 in adipose tissue may reduce lipid influx into the liver, resulting in the reduction of hepatic lipid accumulation.

### Application

In this study, authors show that GLP-1 improves hepatic steatosis in the HFD-induced nondiabetic NAFLD model, which seems to be mediated by enhanced lipolysis with increased systemic energy expenditure. These actions of GLP-1 would be ideal for the treatment of human disease of NAFLD, with or without diabetes.

### Terminology

NAFLD is a chronic liver disease that is characterized by steatosis that is histologically similar to that in alcoholic liver injury, without excessive alcoholic intake or hepatitis viral infection. NASH, a severe stage of NAFLD, frequently progresses into liver cirrhosis and hepatocellular carcinoma. GLP-1, an incretin hormone produced by intestinal L cells, is an effective therapeutic agent for type 2 diabetes mellitus but is immediately degraded by dipeptidyl peptidase-4 (DPP4). Exenatide, a GLP-1 receptor agonist, has a longer half-life and enhanced potency compared with GLP-1 because it is less susceptible to degradation by DPP4.

### Peer review

The authors focused on a different point, *i.e.*, adipose tissue to elucidate the mechanism of improving NAFLD by GLP-1. This unique study related the enhanced lipid metabolism of adipose tissue by GLP-1 with improvement of NAFLD.

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BRIEF ARTICLE

# *ATG16L1* and *NOD2* polymorphisms enhance phagocytosis in monocytes of Crohn's disease patients

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## Abstract

**AIM:** To investigate if the presence of relevant genetic polymorphisms has effect on the effectual clearance of bacteria by monocytes and granulocytes in patients with Crohn's disease (CD).

**METHODS:** In this study, we assessed the differential responses in phagocytosis by measuring the phagocytic activity and the percentage of active phagocytic monocytes and granulocytes in inflammatory bowel disease patients as well as healthy controls. As both autophagy related like 1 (*ATG16L1*) and immunity-related guanosine triphosphatase gene are autophagy genes associated with CD and more recently nucleotide-binding ligomerization domain-containing protein 2 (*NOD2*) has been identified as a potent inducer of autophagy we genotyped the patients for these variants and correlated this to the phagocytic reaction. The genotyping was done with restriction fragment length polymorphisms analysis and the phagocytosis was determined with the pHrodo™ *Escherichia coli* Bioparticles Phagocytosis kit for flowcytometry.

**RESULTS:** In this study, we demonstrate that analysis of the monocyte and granulocyte populations of patients with CD and ulcerative colitis showed a comparable phagocytic activity (ratio of mean fluorescence intensity) between the patient groups and the healthy controls. CD patients show a significantly higher phagocytic capacity (ratio mean percentage of phagocytic cells) compared to healthy controls (51.91% ± 2.85% *vs* 37.67% ± 7.06%, $P = 0.05$). The extend of disease was not of influence. However, variants of *ATG16L1* (WT: 2.03 ± 0.19 *vs* homozygoot variant: 4.38 ± 0.37, $P < 0.009$) as well as *NOD2* (C-ins) (heterozygous variant: 42.08 ± 2.94 *vs* homozygous variant: 75.58 ± 4.34 ($P = 0.05$) are associated with the phagocytic activity in patients with CD.

**CONCLUSION:** Monocytes of CD patients show enhanced phagocytosis associated with the presence of *ATG16L1* and *NOD2* variants. This could be part of the pathophysiological mechanism resulting in the disease.



**Key words:** Inflammatory bowel disease; Phagocytosis; Polymorphism; Monocytes; Granulocytes; Nucleotide-binding ligomerization domain-containing protein 2; Immunity-related guanosine triphosphatase gene; Autophagy related like 1

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## INTRODUCTION

Inflammatory bowel disease (IBD) is comprised of two major disorders: ulcerative colitis (UC) and Crohn's disease (CD). IBD is a complex genetic and immunological disease, wherein antigens in the lumen of the gut initiate an inadequate immune response in a genetically susceptible host. In normal healthy individuals the immune response to commensals in the intestine is kept under strict regulation. When these regulatory mechanisms fail, for instance when bacterial clearance is impaired, an inflammatory response in the intestines can result in IBD. For effective extracellular bacterial clearance there should be accurate phagocytic activity in the gut by phagocytes like monocytes, macrophages, dendritic cells (DC) and granulocytes. Mononuclear phagocytes are able to present bacterial-derived antigens after phagocytosis *via* major histocompatibility complex (MHC) class II complex to $CD4^+$ T cells to initiate an adaptive immune response. However, several pathogens are capable of evading this mechanism and survive in the cytoplasm. For intracellular bacterial clearance there exists a similar efficient pathway of antigen delivery for MHC class II presentation called (macro) autophagy[1,2]. This is a complex cellular process, present in all eukaryotic cells in which intracellular components including organelles but also bacteria, are sequestered in double-membrane vesicles or vacuoles called autophagosomes that eventually fuse with lysosomes, resulting in the degradation of their contents.

In 2001 *NOD2* was the first gene to be identified as being associated with CD. The discovery led to extensive genetic research of this gene[3]. Nucleotide-binding ligomerization domain-containing protein 2 (*NOD2*) encodes an intracellular receptor that recognizes the bacterial component muramyl dipeptide (MDP) of both gram positive and negative bacteria. *NOD2* thus detects invading pathogens and plays a central role in the production of cytokines and antimicrobial peptides[4] *via* the receptors for inacitive C-kinase/phosphatidylinositol-4,5-bisphosphate, nuclear factor kappa B and mitogen-activated protein kinases[5]. Three major *NOD2* variants are associated with CD: R702W, G908R and L1007fsinsC. The latter, being a frameshift mutation, causes an incapability of transcription activation in response to MDP. It has also been shown that this mutation is involved in other functional abnormalities such as enhanced cytokine expression[6] and decreased expression of interleukin (IL)-10[7]. It has been shown in several studies that in cells of patients carrying the *NOD2* mutations, there is an impaired immune response to microbial infections and bacterial ligands and thus a loss of function. Furthermore, *NOD2* deficiency in mice caused exaggerated intestinal inflammation as a result of disrupted immune responses[8,9].

The genome-wide association studies performed in the first half of this decade, have identified over 30 susceptibility loci that are independently associated with CD, of which two are involved in autophagy[10-13]. One of the strongest associations was found in the variant of the autophagy related like 1 (*ATG16L1*) gene[14]. *ATG16L1* encodes an autophagy pathway protein that forms a non-covalent protein complex with ATG5 and ATG12 (800 kD). This complex is essential for the autophagosome formation. A threonine to alanine substitution at position 300 (T300A) of the WD domain is associated with CD. The second autophagy gene associated with CD is Immunity-related GTPase family M protein (*IRGM*)[15]. *IRGM* belongs to a family of genes encoding interferon-inducible guanosine triphosphatases involved in newly recognised forms of pathogen clearance. The single-nucleotide polymorphisms (SNPs) associated with CD in *IRGM* are located in the flanking region of the gene[16]. Sequencing of the gene in both CD patients as well as healthy controls did not identify any non-synonymous variation in linkage disequilibrium with the associated allele. It has been suggested by Xavier *et al*[17] that susceptibility to CD could operate *via* modulation of *IRGM* gene expression. Because dysregulated host responses to intracellular organisms could contribute to the development of CD we hypothesize that one of the underlying mechanisms of this inadequate response is an impaired innate immune response showing in either a disabled or overly active phagocytic uptake of antigens by granulocytes and monocytes. It has recently been shown that there is a physical interaction between *NOD2* and *ATG16L1* and that this interaction is required for autophagic clearance of intracellular pathogens[18]. In this study we first assessed the differential responses in phagocytosis by measuring phagocytic activity and the percentage of active phagocytic monocytes and granulocytes in IBD patients as well as healthy controls (HC). Secondly, we correlated phagocytic capacity to the known associated variant in *ATG16L1*, *IRGM* and *NOD2* (C-ins). This revealed an impaired phagocytic reaction in IBD patients that carried the mutant alleles.

## MATERIALS AND METHODS

### Patients and electronic data collection

In this study 99 IBD patients (65 CD and 34 UC) were included, along with 8 healthy controls. All patients were recruited through the outpatient clinic at the department of Gastroenterology of the Academic Medical Centre (AMC) Amsterdam, the Netherlands as part of the Elephant Study. This study was initially designed to associate the immunological phenotype of IBD patients to the genotype of the known susceptibility genes and clinical phenotype. All clinical phenotypic patient data is available in an electronic patient file, part of which is

**Table 1 Clinical phenotypic characteristics of study population *n* (%)**

| | CD (*n* = 65) | UC (*n* = 34) |
|---|---|---|
| Gender: M/F | 29/36 | |
| Montreal classification | | |
| Age at diagnosis (yr) | 27 (9-68) | |
| < 17 | 10 (15.4) | |
| 17-40 | 44 (67.7) | |
| > 40 | 9 (13.8) | |
| Disease localisation | | |
| Terminal ileum (L1) | 17 (26.2) | |
| Colonic (L2) | 8 (12.3) | |
| Ileocolonic (L3) | 23 (35.4) | |
| Upper gastrotractinal (L4) | 0 | |
| Disease behavior | | |
| Non-stricturing /non-penetrating | 20 (30.8) | |
| Stricturing | 15 (23.1) | |
| Penetrating | 11 (16.9) | |
| Missing | 19 (29.2) | |
| Age at diagnosis (yr) | | 32 (19-74) |
| < 40 | | 25 (73.5) |
| > 40 | | 7 (20) |
| Disease localisation | | |
| Proctitis (L1) | | 3 (8.8) |
| Left sided (L2) | | 11 (32.4) |
| Pancolitis (L3) | | 10 (29.4) |
| Missing | | 10 (29.4) |
| Disease activity (Y/N) | 17/47 | 14/10 |
| Positive family history | 12 | 8 |
| Operated on | 40 | 7 |

CD: Crohn's disease; UC: Ulcerative colitis; M/F: Male/female; Y/N: Yes/no.

excerpted and listed in Table 1. All patients and controls gave informed consent and the Elephant Study was approved by the ethics review committee of the AMC.

### Genotyping with restriction fragment length polymorphisms

The Elephant Study cohort was genotyped for three of the CD-associated genes *ATG16L1*, *IRGM* and *NOD2*. DNA was isolated from venous blood, which was collected within the framework of the Elephant Study. Genotyping for the SNPs was performed by polymerase chain reaction restriction fragment length polymorphisms assay. Designed primers, thermal cycling and restriction enzymes (New England BioLabs, Ipswich, MA, United States) are listed in Table 2. Restriction fragments were separated and visualised using 3% agarose gel containing ethydium bromide.

### Phrodo assay and flow cytometry

The pHrodo™ *Escherichia coli* BioParticles® Phagocytosis Kit for Flow Cytometry from Invitrogen Molecular Probes (Eugene, OR, United States) was used to assess the phagocytic capacity of our Elephant Study cohort and HCs. The dye is nonfluorescent at neutral pH and bright red fluorescent in acidic environments. The advantage of this is that actual phagocytosis and lysosomal acidification is measured while extracellular adherent particles are not detected. The pHrodo™ *Escherichia coli* BioParticles® Phagocytosis assay was performed according to the manufacturer's protocol using heparinized blood from all included patients and HCs. Granulocytes and monocytes were discriminated on the basis of their forward and site scatter profiles by flow cytometry using the FACSscan from BD Biosciences (Erembodegem, Belgium). Phagocytic activity of monocytes and granulocytes was measured as the ratio of the mean of the mean fluorescent intensity of the positive control at 4 ℃ compared to the one at 37 ℃. Percentage phagocytic cells were calculated using the ratio of gated cells in M1 at 4 ℃ and 37 ℃.

### Statistical analysis

Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, United States) or GraphPad (GraphPad Software Inc., La Jolla, CA, United States). All comparisons with phagocytic activity and the percentage of phagocytic cells were tested between the different groups using non-parametric Mann-Whitney and Kruskall-Wallis tests. Significance level was set at 0.05. Association of the different genotypes with the phagocytic activity or cells was tested using a one-way ANOVA and linear regression analysis. *P*-values of 0.05 or less were considered significant.

## RESULTS

### Phagocytosis in granulocytes and monocytes in IBD patients

To test for phagocytic capacity in both granulocytes and monocytes the phagocytic activity and the percentage of phagocytic cells was measured by flow cytometry. Analysis of both the granulocyte population and the monocyte population showed a comparable phagocytic activity between the patient group and the healthy controls (Figure 1). When comparing the amount of active phagocytic granulocytes of both the IBD patient group and the healthy controls, similar results were obtained for both groups (Figure 2). Interestingly, a significant difference was found in the percentage of active phagocytic monocytes of IBD patients when compared to the healthy controls ($P < 0.04$). When comparing all three groups a significant difference was found between the CD patient group and the healthy controls ($P = 0.05$) (Figure 2). A similar pattern was seen in the group of the UC patients when compared with the healthy controls. As expected, both CD and UC patient groups showed a comparable percentage of active phagocytic monocytes and granulocytes (see Figure 2 and data not shown).

### Phagocytosis correlated with disease activity

Since monocytes display an enhanced amount of active phagocytic cells in comparison with the healthy controls, disease activity and possibly the extend of the disease might be of influence. Disease activity was defined as the patient having a leucocytosis, elevated C-reactive

**Table 2 Designed primers and restriction enzymes for genotyping by restriction fragment length polymorphisms**

| Gene | Forward | Reverse | Restriction enzyme |
|---|---|---|---|
| *ATG16L1* rs2241880 | GGTACCCTCACTTCTTTACCAGAA CCAGGAAGAG | TGGAGTCCACAGGTTAGTGTGCAGGAGAGTAAGG | *Sap*1 |
| *IRGM* rs13361189 | CCCGTGTCGTACCCAAGCAGAGTGTGCTTGAAGA | CTTTACCATTGTACTCCTTGTGCCCAGCAGGTG | *Mbo*I |
| *NOD2* rs2066847 rs5743293 | ATGTGTCTAAGGGACAGGTG | AACTGAGGTTCGGAGAGCTA | *Nla*IV |

rs2066847 and rs5743293 are the same single-nucleotide polymorphism (SNP) in nucleotide-binding ligomerization domain-containing protein 2 (*NOD2*). *ATG16L1*: Autophagy related like 1; *IRGM*: Immunity-related GTPase family M protein.

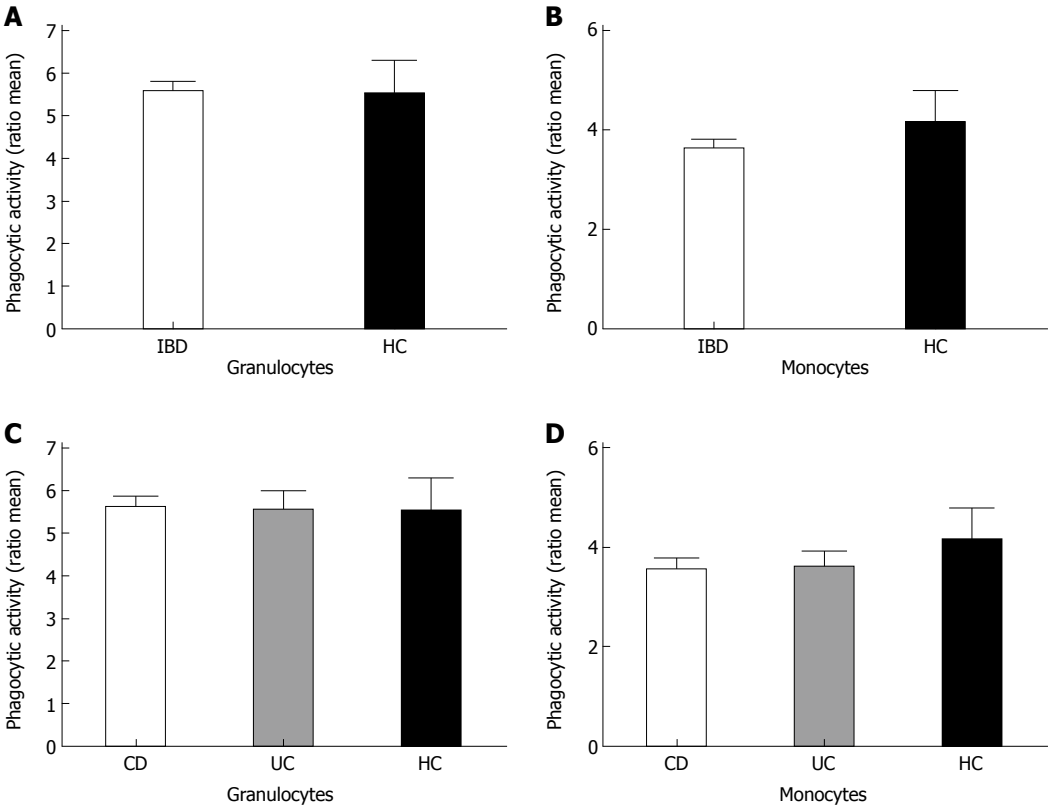


**Figure 1 Phagocytic activity of the disease groups compared to healthy controls.** Bars represent ratio of the mean phagocytic activity. Total inflammatory bowel disease patients (IBD) patients (A, B for granulocytes and monocytes respectively) were analyzed as well as for separate Crohn's disease (CD) and ulcerative colitis (UC) patients compared to healthy controls (C, D). HC: Healthy controls.

protein level and clinical view of the gastroenterologist. The extent of the disease was established and defined using the Montreal Classification (Table 1). Analysis of the influence of the disease activity on phagocytic activity did not reveal any differences between the different categories in UC or CD (Figure 3). The extent of the disease also does not influence on both the phagocytic activity and the percentage of active phagocytic cells (results not shown).

### *Phagocytosis associated with variants in IRGM, ATG16L1 and NOD2*

We next investigated whether the genotypes of our CD patient cohort for *ATG16L1*, *IRGM* and *NOD2* were associated with the overall phagocytic activity and the percentage of active phagocytic cells (Figure 4). Figure 4C shows a positive association of the variants of *ATG16L1* and the phagocytic activity of the monocytes ($P < 0.009$). A *P*-value of 0.08 was found for the amount of active phagocytic monocytes when all three groups were compared. Although we do see the same trend for the variant in *IRGM*, no significant association has been found (Figure 4A, B). The homozygous mutant genotype of the variant 3020 C-ins of *NOD2* influences both phagocytic activity and the percentage of active monocytes, the latter showing a significant difference ($P = 0.05$)

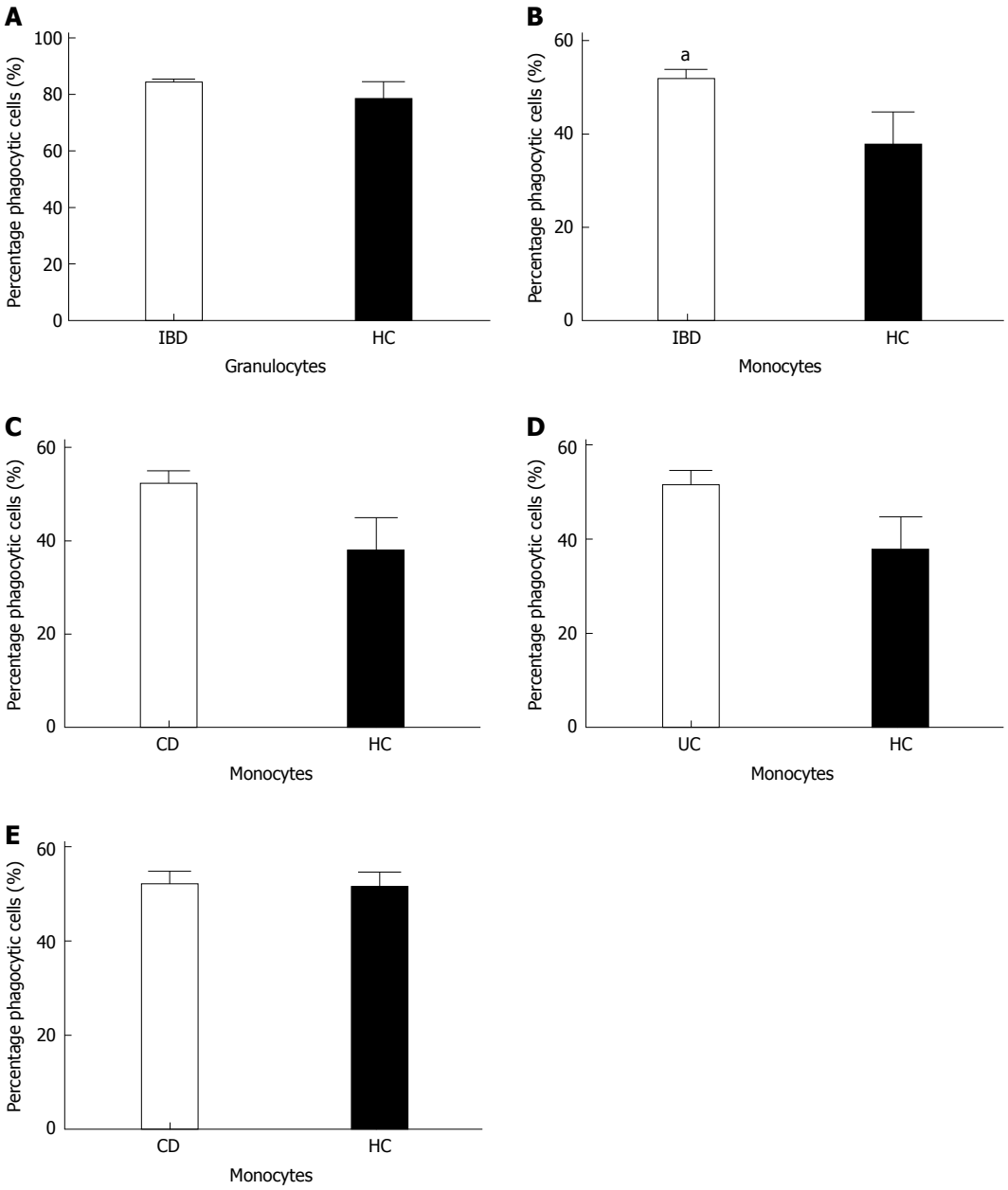


**Figure 2 Percentage of phagocytic cells in inflammatory bowel disease patients patients and healthy controls.** A: Percentages of active granulocytes are comparable between both groups; B: Significantly higher amount of active phagocytic monocytes was found in the inflammatory bowel disease (IBD) patient group compared to healthy controls ($P = 0.0408$), $^{a}P < 0.05$ *vs* HC group; C: Crohn's disease (CD) patients show a significantly higher percentage of active phagocytic monocytes compared to healthy controls; D, E: This was not seen when compared to ulcerative colitis (UC) patients. Bars represent means.

(Figure 4E, F).

## DISCUSSION

Monocytes are of importance in extracellular bacterial clearance and therefore play a role in the regulation of the innate immune response of IBD patients. We demonstrate that monocytes of CD patients show enhanced phagocytosis. In addition we show that the enhanced phagocytosis is not influenced by disease activity and is associated with the disease-related variants of both *ATG16L1* and *NOD2*. The *ex vivo* model we use gives us the opportunity to study the functional consequences of polymorphisms in IBD approximate to the natural situation. The increased phagocytosis in CD patients can result in an accumulation of bacterial products in the cell and secondarily lead to an increased inflammatory reaction.

Phagocytosis and autophagy are processes that are of major importance regarding bacterial clearance. Phagocytosis by professional phagocytes like macrophages, monocytes and granulocytes is responsible for clearing the extracellular compartment; autophagy plays its role in intracellular bacterial clearance. Just a few years ago it has been shown that impaired bacterial clearance is a potential pathogenic factor in IBD. Monocytes are immediate effector cells and produce cytokines and perform

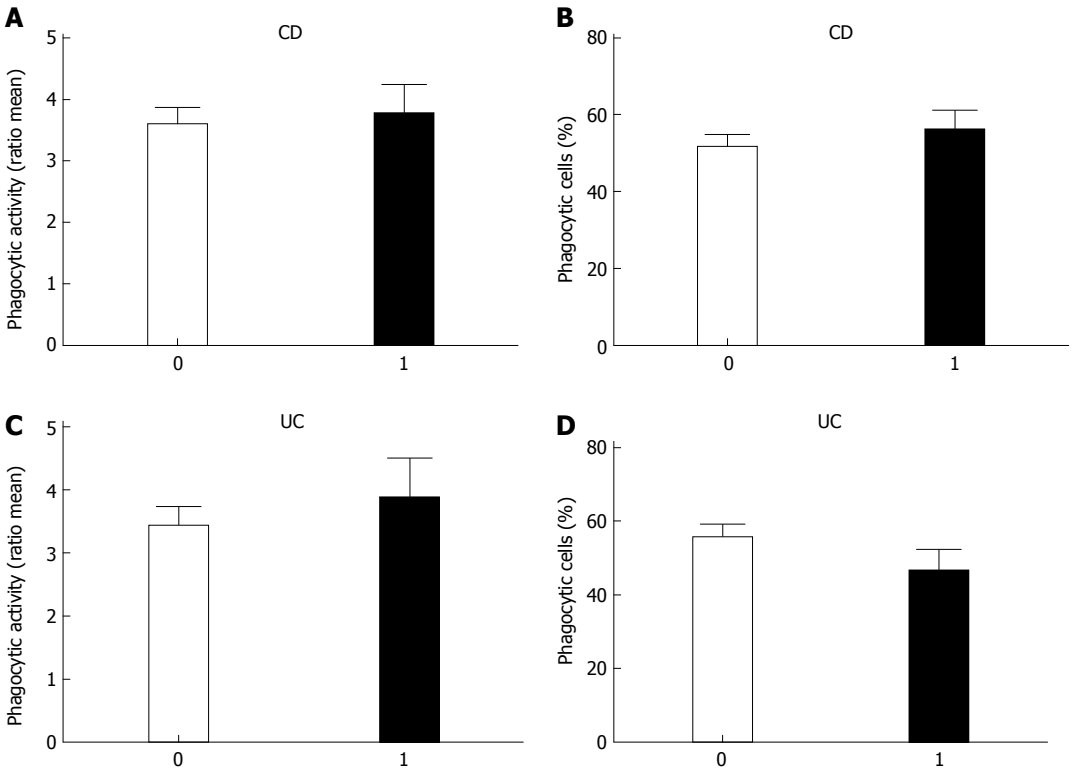


**Figure 3 Phagocytic activity and percentage of phagocytic cells and the correlation with disease activity in Crohn's disease patients and ulcerative colitis patients.** A, B: Crohn's disease (CD); C, D: Ulcerative colitis (UC). 0: No disease activity; 1: Disease activity.

phagocytosis during inflammation[19,20]. Monocytes derive from a myeloid precursor and in mice two subsets of monocytes, Ly-6C$^+$ and Ly-6C$^-$, leave the bone marrow to enter the blood. The Gr1$^+$/Ly-6C$^+$ monocytes give rise to macrophages and DCs during different infections, such as *Listeria monocytogenes* and *Toxoplasma gondii,* but are also found in tumors as suppressor cells, as well as contribute to the recovery of spinal cord injury. Monocytes can therefore have activating as well as inhibiting functions in the immune response[20].

Not only genes involved in primary recognition of bacterial compounds such as *NOD2*, but also genes involved in autophagy were found to be of interest due to the genome-wide association studies, which identified several autophagy genes as susceptibility genes for CD. Autophagy serves its purpose in the innate immune response by clearing several intracellular pathogens, such as *Salmonella enterica*, *Mycoplasma tuberculosis* and *Listeria monocytogenes*[16]. When this system fails, pathogens are able to expand and an adaptive response should start to remove these pathogens. The failure of the innate response, called the theory of innate immune deficiency, has been proposed as responsible for the elevated immune activation seen in CD patients carrying this SNP[21]. This is in concordance with our data. Patients carrying the mutant allele have higher phagocytic activity ($P < 0.009$). This can be explained by the fact that when the innate immune response is inadequate, because of diminished autophagy due to impaired autophagosome formation, the adaptive immune response is overactivated, resulting in a higher percentage of activated monocytes. However, this does not mean that these monocytes have a more effective phagocytic capacity, measured in phagocytic activity. This was shown to be somewhat higher in the healthy controls. Two other studies looked at the phagocytosis of monocytes in relation to *NOD2* polymorphisms. Henckaerts *et al*[22] showed that *NOD2* variants were associated with reduced phagocytosis and bacteremia in critically ill patients and Glubb *et al*[23] demonstrated that *NOD2* was shown to affect the elimination of the *Mycobacterium avium* subspecies *paratuberculosis* from peripheral blood monocytes, whereas *ATG16L1* polymorphism showed increased expression of IL-10 and IL-6. Concerning the effect of the *ATG16L1* polymorphism on cytokine production, Plantinga *et al*[24] demonstrate that the genetic variation in *ATG16L1* is associated with higher production of pro-inflammatory cytokines (IL-1β and IL-6) upon *NOD2* stimulation in CD patients that could drive the chronic inflammation.

A physical interaction between *NOD2* and *ATG16L1* appears to be essential for autophagic clearance of intracellular pathogens; *NOD2* seems to be an autophagy inducer[18,25]. Our data are in accordance with this observation. We have shown that IBD patients carrying both risk alleles show a significantly higher phagocytic capacity in both *ATG16L1* and *NOD2*. In the case of *ATG16L1* this can be due to loss of response showing that carrying one or more risk alleles interferes with autophagy and enhances the phagocytic capacity as a back-up mechanism. The same holds true for the *NOD2* vari-

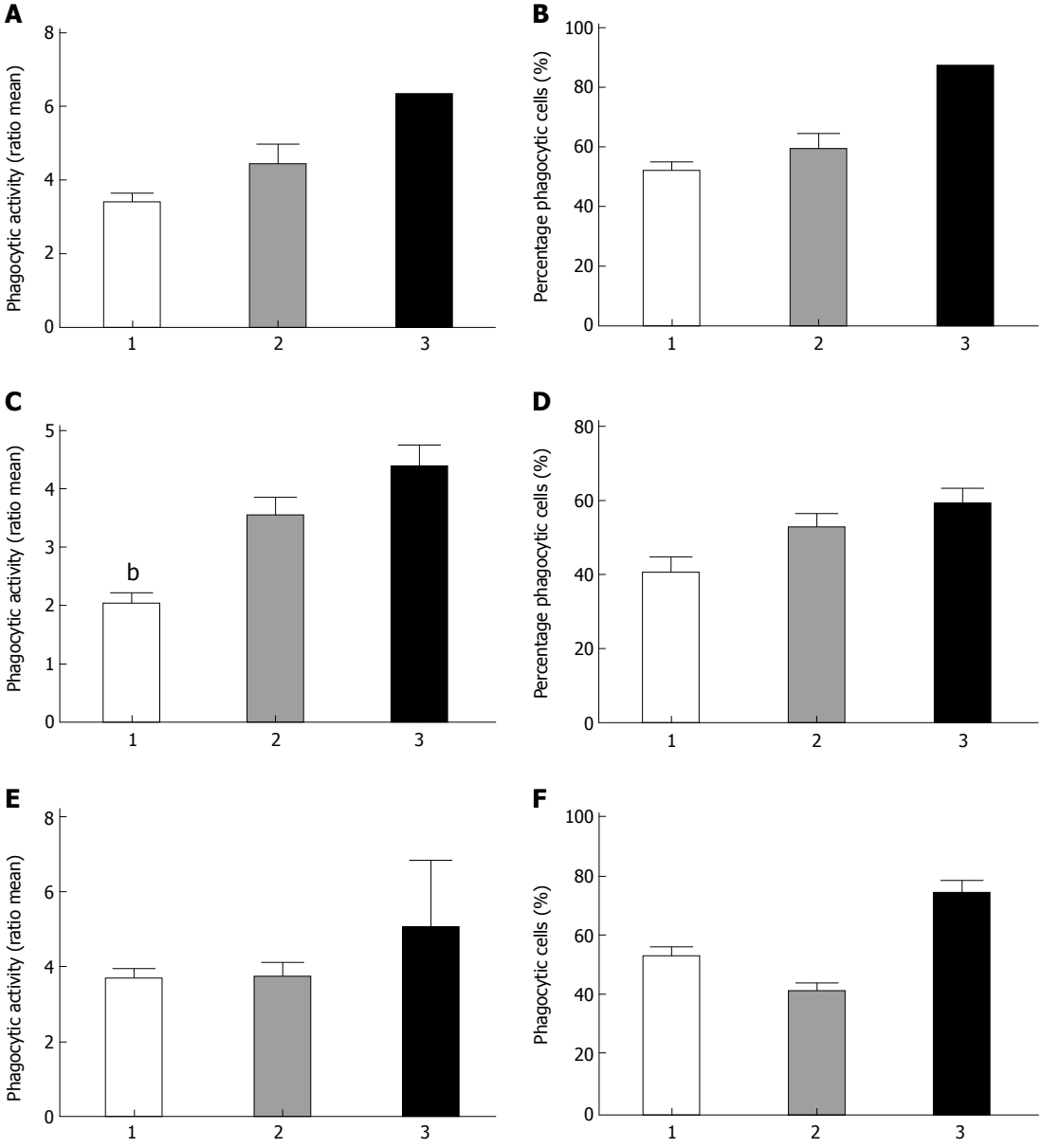


**Figure 4 Phagocytosis and variants in Crohn's disease associated genes autophagy related like 1, immunity-related guanosine triphosphatase gene and nucleotide-binding ligomerization domain-containing protein 2.** A, B: Genotypes in immunity-related guanosine triphosphatase variant and association with either phagocytic activity or the percentage of active monocytes, respectively; C, D: Genotypes of the autophagy related like 1 variant associated with enhanced phagocytic activity ($P = 0.009$) and percentage of active monocytes respectively. [b]$P < 0.01$ *vs* genetype 3; E, F: The genotype of the variant 3020 C-ins of nucleotide-binding ligomerization domain-containing protein 2 and phagocytic activity as well as the percentage of active monocytes ($P = 0.05$) respectively. Genotypes 1: Wild type; Genotypes 2: Heterozygous variant; Genotypes 3: Homozygous variant.

ant as this causes a loss of function. When patients carry this risk allele they have a dysfunctional protein causing a direct problem in the formation of the autophagosome, thereby impairing autophagy. In this case an enhanced phagocytic capacity is also observed, strengthening the hypothesis of a backup mechanism. Our data also demonstrate the same trend in the patients who carry the variety in *IRGM* ($P < 0.08$), however the sample size was too small to show significant differences. Therefore, future studies with a higher number of participants must be undertaken to explore the role of *IRGM* in phagocytosis of monocytes.

## COMMENTS

### Background

Single-nucleotide polymorphisms (SNPs) in susceptibility genes in inflammatory bowel disease (IBD) can contribute to the disease. The autophagy related like 1 (*ATG16L1*) and nucleotide-binding ligomerization domain-containing protein 2 (*NOD2*) susceptibility genes and their role in autophagy and elimination of intracellular bacteria are extensively studied. Most studies were done in mice and cell lines. Authors demonstrate that in humans with variants of *ATG16L1* and *NOD2* the clearance of bacteria by phagocytosis is enhanced compared to controls.

### Research frontiers

There are at the moment 163 different SNPs that are associated with IBD described. The focus on the current research is to analyse the relationship be-

tween genotypes and phenotypes for all IBD-associated polymorphisms.

### Innovations and breakthroughs

The analysis of the relationship between genotypes and phenotypes is currently mainly focussed on elucidating the correlation with the clinical phenotype. The research toward the determination of functional differences in the innate and adaptive immune responses that could play a role in the IBD risk is the challenge for the coming years.

### Applications

IBD patients that have the risk alleles *ATG16L1* and/or *NOD2* have a different immunologic reactivity and might benefit from a treatment regimen focused on the specific disease variant effects

### Peer review

The authors present an interesting study showing that in Crohn's disease (CD) patients phagocytic activity of monocytes is enhanced. Moreover they could demonstrate that this increased activity is associated with the genotype. This information is interesting for all of those who try to uncover the pathophysiological mechanism resulting in CD.

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BRIEF ARTICLE

# Effect of branched-chain amino acids in patients receiving intervention for hepatocellular carcinoma

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## Abstract

**AIM:** To investigate the usefulness of branched-chain amino acids (BCAA) before transarterial chemoembolization (TACE) or radiofrequency ablation (RFA).

**METHODS:** We investigated the usefulness of pre-intervention with BCAAs by comparing patients treated with BCAAs at 12.45 g/d orally for at least 2 wk before TACE or RFA and those not receiving such pretreatment. A total of 270 patients with hepatocellular carcinoma complicated by cirrhosis were included in the study. Mean changes from baseline (Δ) in serum albumin (Alb), C-reactive protein (CRP), and transaminase levels, as well as peak body temperature were also determined and compared at days 2, 5, and 10 after the start of TACE or RFA.

**RESULTS:** In patients who underwent TACE or RFA, BCAA pre-intervention significantly suppressed the development of post- intervention hypoalbuminemia and reduced inflammatory reactions during the subsequent clinical course. After TACE, the ΔAlb peaked on day 2, remained constantly lower in BCAA-treated patients, compared to the control group, and was -0.13 ± 0.42 g/dL in BCAA-treated patients and -0.33 ± 0.51 g/dL in untreated patients on day 10. The ΔCRP was also significantly lower in BCAA-treated patients on days 2, 5 and 10 after TACE. Like the trends noted after TACE, a similar tendency was noted as to the ΔAlb and ΔCRP after RFA. The changes in serum Alb level were inversely correlated with CRP changes; therefore, a possible involvement of the anti-inflammatory effect of BCAAs was inferred as a factor contributory to the suppression of decrease in serum Alb level.

**CONCLUSION:** Pre-intervention with BCAAs may hasten the recovery of serum Alb level and mitigate postoperative complications in patients undergoing TACE or RFA.



**Key words:** Branched-chain amino acids; Cirrhosis; Hepatocellular carcinoma; Transarterial chemoembolization; Radiofrequency ablation; Hypoalbuminemia

**Core tip:** This study investigated whether the short-term effect of branched-chain amino acid (BCAA) for transarterial chemoembolization (TACE) or radiofrequency ablation (RFA), focusing on BCAA treatment prior to therapeutic intervention, suppresses the decrease in serum albumin (Alb) level associated with TACE or RFA and reduces postoperative complications. We thought

that C-reactive protein (CRP) elevation was suppressed in BCAA-treated patients, that the severity of fever was milder as a result of BCAA treatment, and that the serum Alb level decreased to a lesser extent in patients with milder changes in CRP level.

Ishihara T, Iwasa M, Tanaka H, Kaito M, Ikoma J, Shibata T, Takei Y. Effect of branched-chain amino acids in patients receiving intervention for hepatocellular carcinoma. *World J Gastroenterol* 2014; 20(10): 2673-2680 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i10/2673.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i10.2673

## INTRODUCTION

Decrease in plasma albumin (Alb) level occurs early after therapeutic intervention such as transarterial chemoembolization (TACE) and radiofrequency ablation (RFA) for treatment of hepatocellular carcinoma (HCC), and the underlying mechanism of this condition includes the extravasation of Alb into the embolized or ablated hepatic region and the involvement of inflammatory cytokine[1]. HCC arises in association with cirrhosis in most patients, readily produces hypoalbuminemia, edema, ascites, and impairs the patient's quality of life, with a poor vital prognosis[2,3]. Measures to cope with hypoalbuminemia are needed in that therapeutic intervention options as well as the prognosis in HCC patients are dependent on this condition[4-7].

When administered to cirrhosis patients presenting with hypoalbuminemia, the branched-chain amino acids (BCAA) efficiently increase the Fisher ratio and thus improve hypoalbuminemia[8-13]. Improved event-free survival rate has been reported in patients with liver cirrhosis maintained on long-term continuous BCAA treatment[14,15], and the use of such treatment is recommended in guidelines in various countries[16]. In recent years, a liver-protective effect, *e.g.*, lessening of oxidative stress, of BCAAs has been attracting attention[17-19] and BCAA treatment has been reported to maintain skeletal muscles; these multifunctional pharmacological effects of this preparation have become the focus of increased interest[4].

Hypoalbuminemia is noted often early after TACE or RFA in patients with HCC complicated by cirrhosis[1]. This retrospective study was conducted to determine whether pre-intervention with BCAAs is effective in suppressing such hypoalbuminemia early after the intervention, as well as factors involved in such effect.

## MATERIALS AND METHODS

A total of 270 patients with HCC complicated by cirrhosis who were admitted to Yokkaichi Digestive Disease Center and treated there between April 2004 and April 2012 were included in the study. TACE was performed in 162 patients (of them, 76 and 86 patients were treated with BCAAs and untreated before TACE, respectively) and RFA in 108 patients (of them, 40 and 68 patients were treated with BCAAs and untreated before RFA, respectively). Cirrhosis and HCC were diagnosed based on the findings in various imaging studies and hematological/blood chemical test results, and, where deemed necessary, liver biopsy and/or tumor biopsy findings were also used. Anticancer agents, cisplatin and epirubicin hydrochloride, were used in patients who underwent TACE. In the BCAA-treated group, patients received LIVACT® Granules (Ajinomoto Pharma Co., Ltd., Tokyo) at 12.45 g/d orally (in 3 divided doses daily, after each meal) for at least 2 wk before TACE or RFA, and the oral dosage of the drug remained unchanged throughout the study period. Patients who had not received such treatment constituted the non-BCAA-treated group (control group). Patients who received transfusion of Alb preparation, blood transfusion, and/or newly instituted BCAA treatment and patients who underwent TACE in combination with RFA during the course of study treatment were excluded from the study.

Patients were compared with respect to the following clinical parameters: pre-treatment (baseline) Child-Pugh score [serum Alb level, prothrombin time (PT) expressed as a percentage, total bilirubin (T-Bil), and severity of ascites/encephalopathy], clinical stage of HCC, maximum tumor diameter, C-reactive protein (CRP) level, and tumor markers. Mean changes from baseline (Δ) in serum Alb, CRP, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels, as well as peak body temperature were also determined and compared at days 2, 5, and 10 after the start of treatment.

### *Statistical analysis*

Data obtained were expressed as the mean ± SD, and comparisons between the patient groups were performed using $\chi^2$ test and *t* test. Changes in laboratory test parameters and body temperature from baseline (Δ) were analyzed using *t* test. Pearson's product-moment correlation coefficient was used for testing the correlation between the two groups.

## RESULTS

### *Patient demographic characteristics of the two groups prior to TACE or RFA*

Table 1 summarizes the demographic characteristics of patients who underwent TACE in this study. Patients in whom cirrhosis was etiologically not attributable to hepatitis virus were more frequent in the control group. There was a greater percentage of patients with advanced (stage-IVa) HCC in the BCAA-treated group although no significant difference was noted between the two groups in respect of maximum tumor diameter. BCAA-treated patients had a lower hepatic functional reserve, as indicated by a serum Alb level of 3.3 ± 0.4 g/dL, than non-BCAA-treated patients with a serum Alb level of 3.8 ± 0.5

**Table 1 Characteristics of study groups underwent transarterial chemoembolization and radiofrequency ablation**

| Characteristics | TACE | | | RFA | | |
|---|---|---|---|---|---|---|
| | Control group *n* = 86 | BCAA group *n* = 76 | *P* value | Control group *n* = 68 | BCAA group *n* = 40 | *P* value |
| Male/female | 57/29 | 46/30 | 0.746 | 47/21 | 13/27 | < 0.001 |
| Age (yr) | 72.2 ± 8.6 | 71.7 ± 7.2 | 0.055 | 71.9 ± 6.5 | 73.0 ± 6.0 | 0.386 |
| Cause of hepatic cirrhosis (B/C/non B non C) | 13/49/24 | 9/64/3 | 0.025 | 7/47/14 | 0/34/6 | 0.068 |
| Child-Pugh (A/B/C) | 11/1/1974 | 35/36/5 | < 0.001 | 59/9/0 | 23/16/1 | < 0.001 |
| Child-Pugh score | 5.6 ± 0.9 | 6.8 ± 1.4 | < 0.001 | 5.7 ± 0.9 | 6.6 ± 1.1 | 0.001 |
| HCC clinical stage (Ⅰ/Ⅱ/Ⅲ/Ⅳa/Ⅳb) | 21/49/11/5/0 | 13/14/21/27/1 | < 0.001 | 21/40/6/0/1 | 16/16/7/1/0 | 0.276 |
| Alb (g/dL) | 3.8 ± 0.5 | 3.3 ± 0.4 | < 0.001 | 3.6 ± 0.4 | 3.2 ± 0.4 | < 0.001 |
| CRP (mg/dL) | 1.0 ± 0.3 | 0.3 ± 0.5 | < 0.001 | 1.0 ± 0.3 | 0.3 ± 0.5 | 0.010 |
| Body temperature (℃) | 36.2 ± 0.4 | 36.4 ± 0.5 | 0.006 | 36.3 ± 0.5 | 36.4 ± 0.4 | 0.006 |
| PT | 95% ± 10% | 86% ± 15% | < 0.001 | 94% ± 10% | 87% ± 14% | 0.008 |
| T-Bil (mg/dL) | 0.9 ± 0.5 | 1.3 ± 1.1 | 0.007 | 0.8 ± 0.3 | 1.0 ± 0.5 | 0.014 |
| AFP (ng/mL) | 392 ± 70 | 1657 ± 434 | 0.009 | 392 ± 70 | 1657 ± 434 | 0.009 |
| PIVKA-Ⅱ (mAU/mL) | 334 ± 975 | 385 ± 1180 | 0.819 | 334 ± 975 | 385 ± 1180 | 0.819 |
| Max tumor size (mm) | 29 ± 26 | 29 ± 32 | 0.937 | 21 ± 8 | 20 ± 8 | 0.364 |

Data were assessed using student's *t* test. HCC: Hepatocellular carcinoma; Alb: Albumin; CRP: C-reactive protein; PT: Prothrombin time; T-Bil: Total bilirubin; AFP: α-fetoprotein; PIVKA-Ⅱ: Prothrombin induced by vitamin K absence-Ⅱ; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation; BCAA: Branched-chain amino acids.

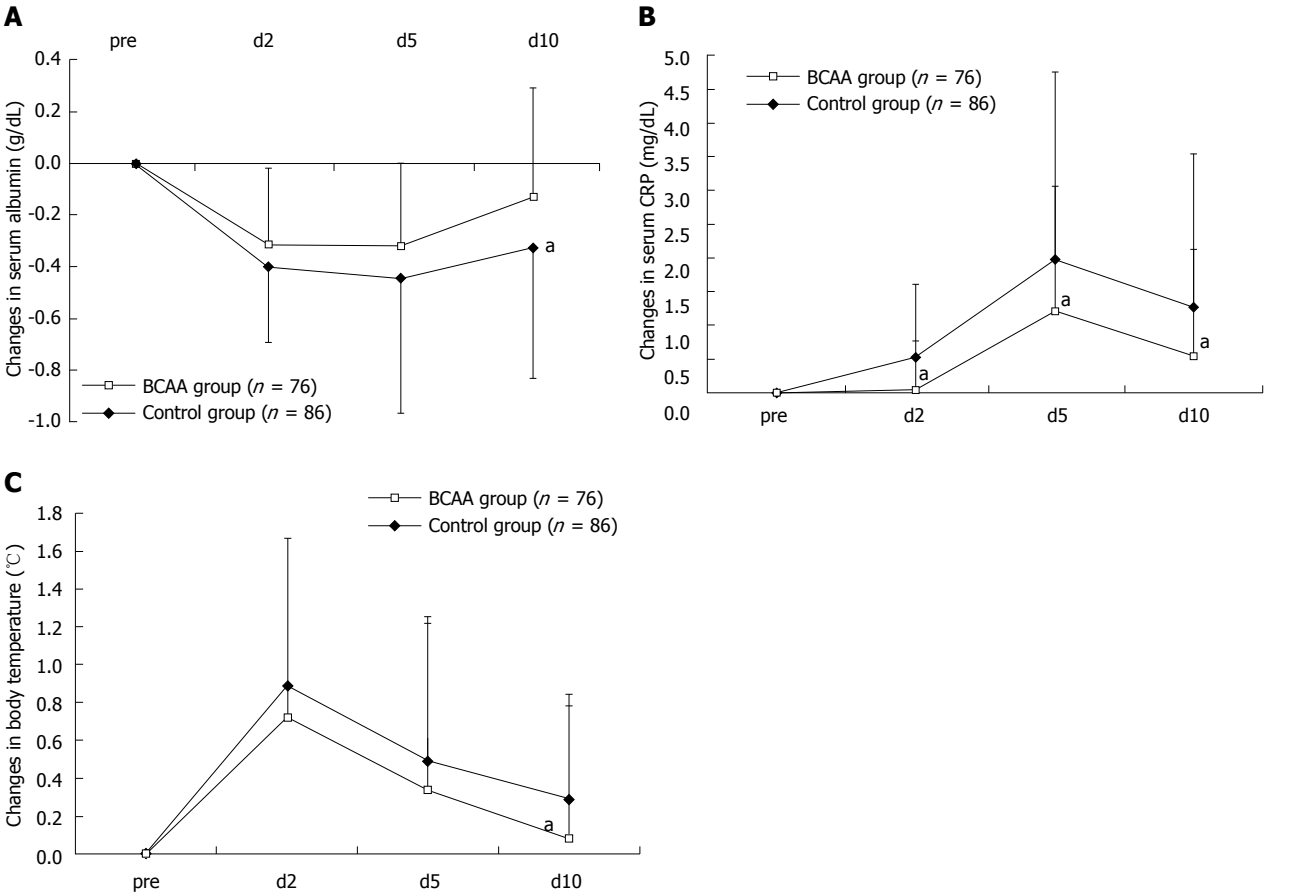


**Figure 1 Changes in serum albumin (A), C-reactive protein (B) and body temperature (C) levels in comparison to the baseline level after transarterial chemoembolization.** Data were assessed using student's *t* test (mean ± SD). $^{a}P < 0.05$ *vs* control group. CRP: C-reactive protein; BCAA: Branched-chain amino acids.

g/dL. PT% and T-Bil were also poorer in BCAA-treated patients; hence the significantly higher Child-Pugh score for these patients. The doses of cisplatin and epirubicin hydrochloride used in combination with TACE did not significantly differ between BCAA-treated and non-BCAA-treated patients. The demographic characteristics

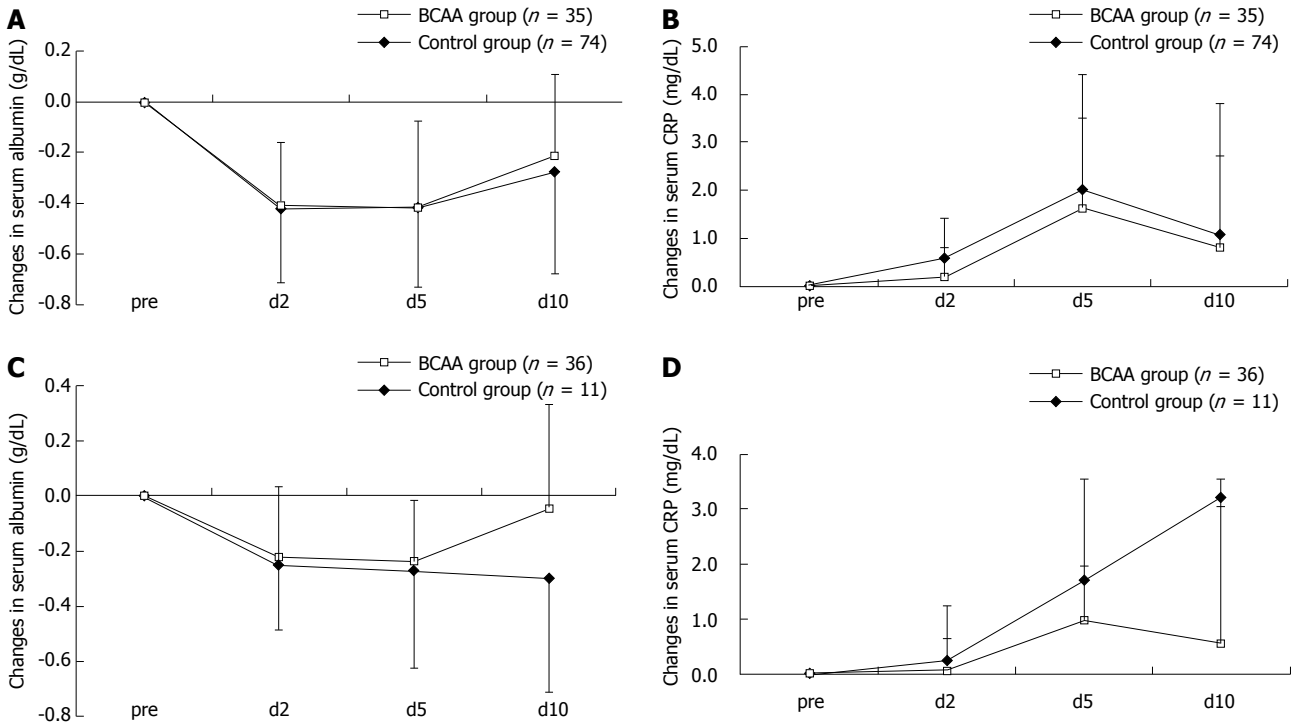


**Figure 2 Comparison in Child A and B patients with serum albumin and C-reactive protein levels after transarterial chemoembolization.** The analysis revealed a tendency for Child A patients to show a faster recovery in serum albumin level (A) and suppression of C-reactive protein (CRP) elevation (B). The analysis revealed a tendency for Child B patients to show a faster recovery in serum albumin level (C) and suppression of CRP elevation (D). Data were assessed using student's *t* test (mean ± SD). BCAA: Branched-chain amino acids.

of patients receiving RFA in this study are shown in Table 1. Depression of hepatic functional reserve, such as a significantly higher Child-Pugh score, was noted for the BCAA-treated group, as was the case with patients who underwent TACE. There was no significant difference between the BCAA-treated and non-BCAA-treated groups with respect to the cause of cirrhosis, clinical stage of HCC, or maximum tumor diameter.

### *Laboratory values and body temperature over time after TACE*

After TACE, the ΔAlb peaked on day 2, remained constantly lower in BCAA-treated patients, compared to the control group, and was -0.13 ± 0.42 g/dL in BCAA-treated patients and -0.33 ± 0.51 g/dL in untreated patients on day 10, thus returning almost to the baseline level in the BCAA-treated group (Figure 1A). The ΔCRP was also significantly lower in BCAA-treated patients on days 2, 5 and 10 after TACE, and peaked on day 5 in both treated and untreated patients (Figure 1B). The maximum body temperature was significantly lower on day 10 post-TACE in the BCAA-treated group (Figure 1C). No significant difference was noted as to ΔAST or ΔALT between BCAA-treated and untreated patients.

As there was a significant difference in the hepatic functional reserve of BCAA-treated and untreated patients before TACE, we carried out an additional analysis with stratification by Child-Pugh A and B. The analysis revealed a tendency for Child A patients to show a faster recovery in serum Alb level and suppression of CRP elevation (Figure 2A and B). Child B patients exhibited the same trends (Figure 2C and D), indicating that responses to BCAA therapy were independent of pre-TACE hepatic functional reserve.

### *Laboratory values and body temperature over time after RFA*

Like the trends noted after TACE, a similar tendency was noted as to the ΔAlb and ΔCRP after RFA; the elevation was suppressed in the BCAA-treated group (Figure 3). The ΔAlb and ΔCRP peaked on days 2 and 5 after RFA, respectively, in these groups, as with the case with these parameters after TACE. As seen in the case of TACE, no significant difference was observed in respect of ΔAST or ΔALT between BCAA-treated and untreated patients.

The stratified analysis of data on patients having undergone RFA also revealed a tendency for the serum Alb level to be recovered earlier and the CRP elevation to be suppressed (the suppression was significant especially in Child B on day 2) in both Child A and Child B patients, again indicating that pre-RFA hepatic functional reserve was unlikely to be involved in the patient response to BCAA therapy (Figure 4).

### *Correlation between ΔAlb and ΔCRP*

The correlation between ΔAlb on day 2 post-TACE and ΔCRP on day 10 post-TACE tended to indicate that the greater the serum CRP elevation, the more decreased was the serum Alb level (Figure 5A). Furthermore, there was a significant negative correlation between ΔAlb on day 2

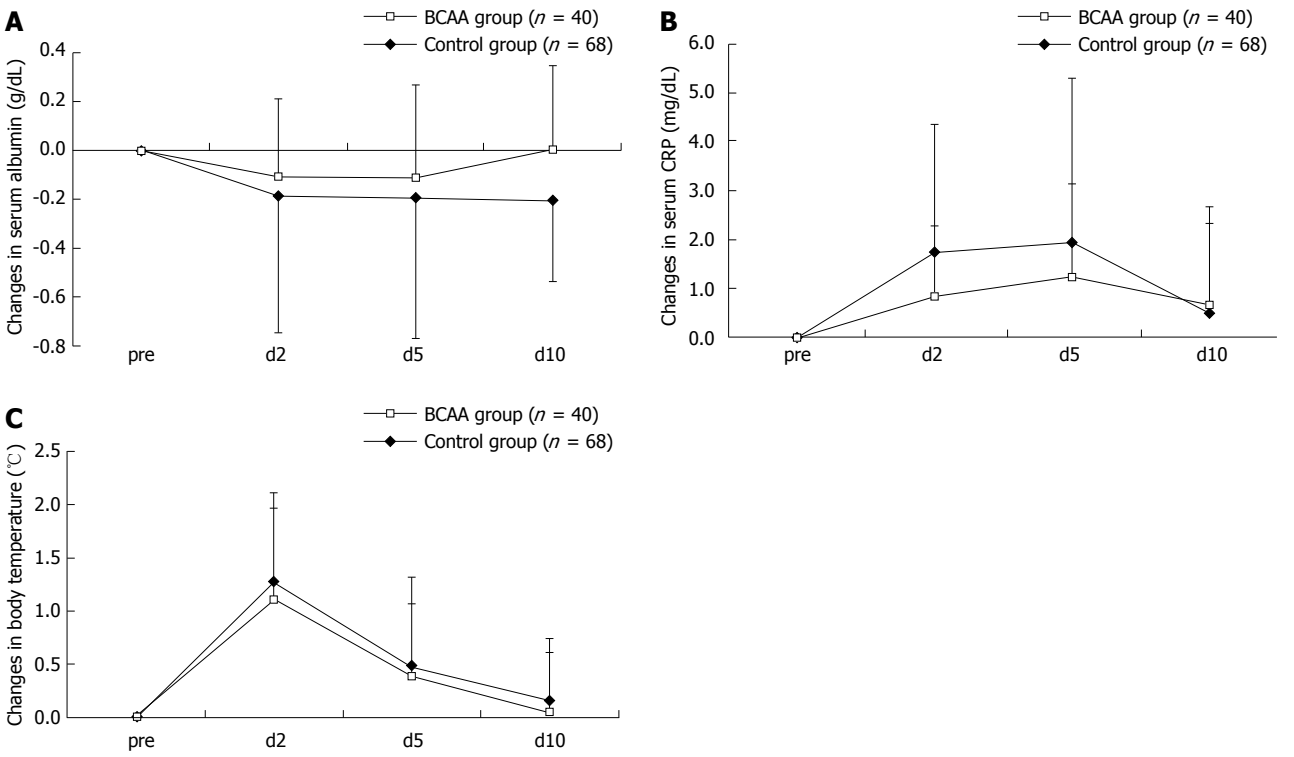


**Figure 3 Changes in serum albumin (A), C-reactive protein (B) and body temperature (C) levels in comparison to the baseline level after radiofrequency ablation.** Data were assessed using student's *t* test (mean ± SD). $^{a}P < 0.05$ *vs* control group. CRP: C-reactive protein; BCAA: Branched-chain amino acids.

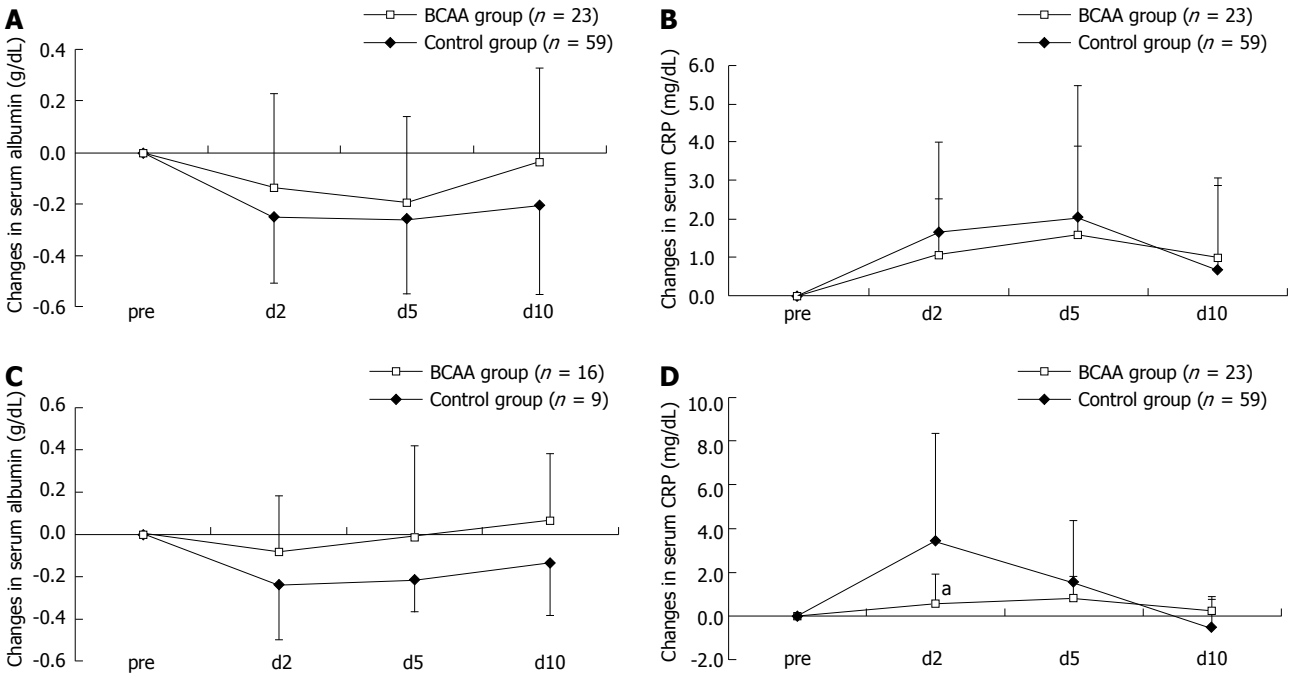


**Figure 4 Comparison in Child A and B patients with serum albumin and C-reactive protein levels after radiofrequency ablation.** A: A faster recovery of serum albumin level was noted in Child A; B: No difference of C-reactive protein (CRP) was noted in changes in Child A; C: A faster recovery of serum albumin level was noted in Child B; D: A significant difference of CRP was noted in changes in Child B. Data were assessed using student's *t* test (mean ± SD). $^{a}P < 0.05$ *vs* control group. BCAA: Branched-chain amino acids.

post-RFA and ΔCRP on day 10 post-RFA (Figure 5B). Analyses for correlation at time points of days 2, 5 and 10 revealed no significant correlation.

## DISCUSSION

TACE and RFA are established treatment modalities for

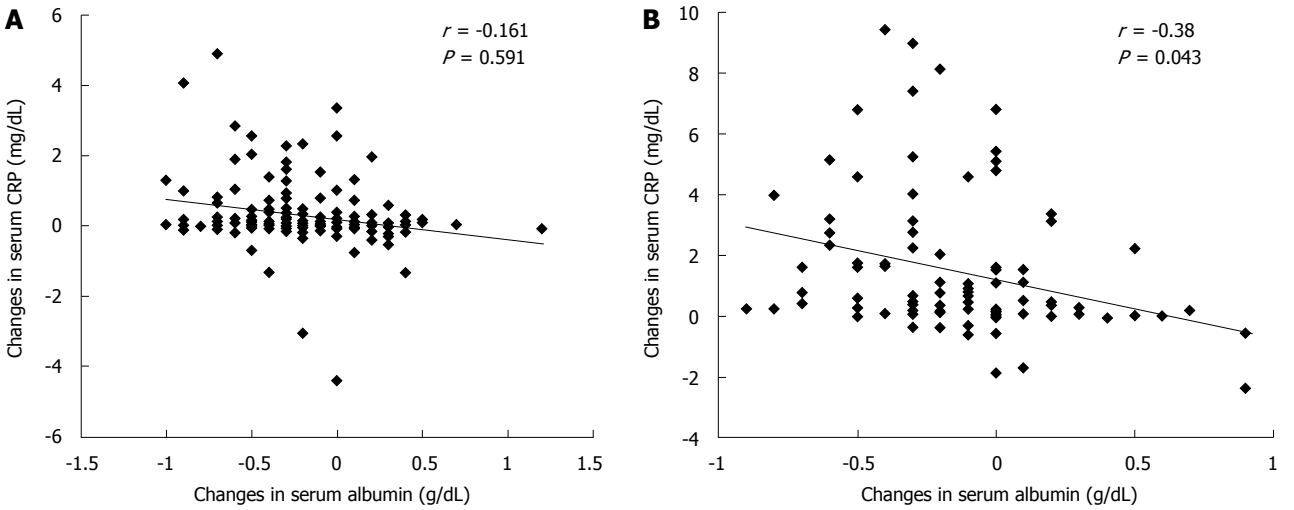


**Figure 5 Relationship between Δalbumin (day 2) and ΔC-reactive protein (day 10).** A: Transarterial chemoembolization; B: Radiofrequency ablation correlation between each variable was tested using Pearson's correlation coefficient.

hepatocellular carcinoma[20]. However, depression of hepatic functional reserve including hypoalbuminemia may develop after these therapeutic interventions[1]. It was reported that treatment with the BCAAs efficiently increases in Fisher ratio and improves hypoalbuminemia in cirrhosis patients presenting with hypoalbuminemia, and such treatment has become extensively used in daily clinical practice[4,5,14,15]. This effect is thought to result from enhanced mRNA translation for Alb with a consequent promotion of protein synthesis *via* activation of the mammalian target of rapamycin (mTOR) of hepatocytes that occurs in response to the BCAA supplement[21,22].

The effect of BCAA treatment on post-TACE hypoalbuminemia has already been investigated; Takeshita *et al*[23] reported that administration of BCAAs at 22:00 after every evening meal inhibited the development of hypoalbuminemia at 2 wk after TACE. Recently, Nishikawa *et al*[24] also reported that BCAA treatment was useful for long-term maintenance of nutrition after TACE in that the development of hypoalbuminemia was found to be suppressed at 1, 3, and 6 mo post-TACE. However, in the former study, nevertheless, patients had newly started BCAA treatment at the time TACE was performed, and the latter report does not refer to the changes during the early stage after TACE; the two reports differ from the present article in these respects.

On the other hand, based on their findings demonstrating a significant correlation between CRP elevation and decrease in serum Alb level, Koda *et al*[1] reported that the underlying mechanism of hypoalbuminemia developed following RFA was as follows: inflammatory cytokines liberated due to inflammation at the sites of cauterization act upon liver cells, thereby inducing production of acute inflammatory reactive proteins such as CRP and inhibiting Alb synthesis. They concluded that BCAA treatment was not effective in reversing a short-term decrease in serum Alb level early after RFA because there was no significant difference in serum Alb level decrease at 1 wk post-RFA between BCAA-pretreated and untreated patients[1].

The results of our review of a number of TACE- or RFA-treated patients demonstrated that the decline in serum Alb level was suppressed over 10 d post-treatment in BCAA-treated patients. BCAA pre-treatment might be effective in suppressing an early decline in serum Alb level after the therapeutic intervention. We inferred that the inflammation-inhibitory effect of BCAA treatment might be involved in the suppression of the decrease in serum Alb level[4,17-19] on the grounds that CRP elevation was suppressed in BCAA-treated patients, that the severity of fever was milder as a result of BCAA treatment, and that the decrease in serum Alb level was also to a lesser extent in patients with milder changes in CRP level. The following mechanism seemed likely to be operating: serum Alb level falls into a trough due to extravasation mainly into the embolized or ablated hepatic region as early as 2 d after TACE or RFA, and in patients receiving BCAA pre-treatment, its anti-inflammatory effect and sustained stimulation of mTOR from continued BCAA treatment lead to serum Alb level restoration nearly to the baseline level by day 10 post-intervention. While a direct hepatocyte damage-mitigating effect of BCAA treatment was demonstrated in an animal study[18,25,26], there was no significant difference in ΔAST or ΔALT between the BCAA-treated and untreated patients in this study; therefore, BCAA treatment is thought to be virtually devoid of hepatocyte damage-mitigating effect in the clinical setting.

The present results suggest that BCAA treatment prior to therapeutic intervention suppresses the decrease in serum Alb level associated with TACE or RFA and may reduce postoperative complications although confirmation of such effect observed in this study through prospective investigation and clarification of the underlying mechanism are needed.

## COMMENTS

### Background

Decrease in plasma albumin (Alb) level occurs early after therapeutic intervention such as transarterial chemoembolization (TACE) or radiofrequency ablation (RFA) for treatment of hepatocellular carcinoma (HCC). Measures to cope with hypoalbuminemia are needed in that therapeutic intervention options as well as the prognosis in HCC patients are dependent on this condition.

### Research frontiers

When administered to patients with cirrhosis, branched-chain amino acids (BCAA) not only raises serum Alb level but also exerts a liver-protective effect such as lessening of oxidative stress. Authors investigated the usefulness of pre-intervention with BCAAs by comparing patients treated with BCAAs for at least 2 wk before TACE or RFA and those not receiving such pretreatment.

### Innovations and breakthroughs

The inflammation-inhibitory effect of BCAA treatment might be involved in the suppression of decrease in serum Alb level on the grounds that C-reactive protein (CRP) elevation was suppressed in BCAA-treated patients, and that the decrease in serum Alb level was also to a lesser extent in patients with milder changes in CRP level.

### Applications

Pre-intervention with BCAAs may hasten the recovery of serum Alb level and mitigate post-operative complications in patients undergoing TACE or RFA.

### Terminology

In recent years, a liver-protective effect, *e.g.*, lessening of oxidative stress, of BCAAs has been attracting attention and BCAA treatment has been reported to maintain skeletal muscles; these multifunctional pharmacological effects of this preparation have become the focus of increased interest.

### Peer review

The study found BCAA pre-intervention could significantly suppress the development of post-operative hypoalbuminemia and reduce inflammatory reactions during the subsequent clinical course in 162 patients, the changes in serum Alb level were inversely correlated with CRP changes. It is very important for patients with HCC to avoid hypoalbuminemia after the treatment of TACE or RFA.

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BRIEF ARTICLE

# Forward-viewing radial-array echoendoscope for staging of colon cancer beyond the rectum

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## Abstract

**AIM:** To evaluate feasibility of the novel forward-viewing radial-array echoendoscope for staging of colon cancer beyond rectum as the first series.

**METHODS:** A retrospective study with prospectively entered database. From March 2012 to February 2013, a total of 21 patients (11 men) (mean age 64.2 years) with colon cancer beyond the rectum were recruited. The novel forward-viewing radial-array echoendoscope was used for ultrasonographic staging of colon cancer beyond rectum. Ultrasonographic T and N staging were recorded when surgical pathology was used as a gold standard.

**RESULTS:** The mean time to reach the lesion and the mean time to complete the procedure were 3.5 and 7.1 min, respectively. The echoendoscope passed through the lesions in 13 patients (61.9%) and reached the cecum in 10 of 13 patients (76.9%). No adverse events were found. The lesions were located in the cecum ($n$ = 2), ascending colon ($n$ = 1), transverse colon ($n$ = 2), descending colon ($n$ = 2), and sigmoid colon ($n$ = 14). The accuracy rate for T1 ($n$ = 3), T2 ($n$ = 4), T3 ($n$ = 13) and T4 ($n$ = 1) were 100%, 60.0%, 84.6% and 100%, respectively. The overall accuracy rates for the T and N staging of colon cancer were 81.0% and 52.4%, respectively. The accuracy rates among traversable lesions ($n$ = 13) and obstructive lesions ($n$ = 8) were 61.5% and 100%, respectively. Endoscopic ultrasound and computed tomography had overall accuracy rates of 81.0% and 68.4%, respectively.

**CONCLUSION:** The echoendoscope is a feasible staging tool for colon cancer beyond rectum. However, accuracy of the echoendoscope needs to be verified by larger systematic studies.



**Key words:** Colon cancer; Neoplasm; Colon; Endoscopic ultrasound; Neoadjuvan therapy; Staging; Forward-

viewing; Colonoscopy

**Core tip:** Endoscopic ultrasound staging of rectal cancer has higher accuracy rate than computed tomography (CT) scan. Unfortunately, with the current design of conventional oblique-viewing radial-array echoendoscope that cannot readily be introduced beyond rectum, staging of colon cancer beyond rectum nowadays depends on results of CT scan. With a design of the novel forward-viewing radial-array echoendoscope that can be easily passed through the entire colon, it was firstly used in this study for staging of colon cancer. The study showed feasibility of the scope and its superiority over CT scan in terms of accuracy rate of colon cancer staging.

Kongkam P, Linlawan S, Aniwan S, Lakananurak N, Khemnark S, Sahakitrungruang C, Pattanaarun J, Khomvilai S, Wisedopas N, Ridtitid W, Bhutani MS, Kullavanijaya P, Rerknimitr R. Forward-viewing radial-array echoendoscope for staging of colon cancer beyond the rectum. *World J Gastroenterol* 2014; 20(10): 2681-2687 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i10/2681.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i10.2681

## INTRODUCTION

Preoperative colon and rectal cancer staging are the main factors in determining the subsequent treatment modality for patients with these types of cancer. Accurate preoperative staging is crucial, as it can greatly influence the results[1]. For rectal cancer, endoscopic ultrasound (EUS) demonstrates a higher accuracy of T and N staging compared to computed tomography (CT). Hence, EUS, rather than CT, was suggested as the staging tool of choice for rectal cancer, and it has had a significant impact on the management of rectal cancer[2]. Consequently, we speculated that if EUS can stage colon cancer beyond the rectum, it may yield more accurate results than CT and may improve the outcomes for colon cancer. Unfortunately, with the current design of EUS, it is not possible to use the EUS to stage colon cancer beyond the rectum. Through the scope, a miniprobe can be used to stage colon cancer; however, only a few studies thus far have reported its accuracy rate. Although it has been used for this purpose for over a decade, the supporting data are not well established[3].

The current radial-array EUS has a limited oblique endoscopic view that precludes deep intubation of the EUS probe into the colon beyond the rectum. With the new design of a forward-viewing radial-array echoendoscope [radial Scan Ultrasonic Video Endoscope EG-530UR2 (FUJIFILM Corporation, Tokyo, Japan) and Ultrasound Processor SU-8000 (FUJIFILM Corporation, Tokyo, Japan)], the scope can readily pass to the cecum to locally stage colon cancer. We pioneered this procedure and initiated this study to report the feasibility and accuracy of the new scope in the T staging of colon cancer, using surgical pathology as a gold standard.

## MATERIALS AND METHODS

From March 2012 to February 2013, patients with colon cancer beyond the rectum identified by colonoscopy in King Chulalongkorn Memorial Hospital, Bangkok, Thailand were eligible for this study. The inclusion criteria included patients aged 18-80 years with colon cancer and with an endoscopic or surgical resection scheduled within 4 wk. The exclusion criteria included patients with contraindications for surgery and/or EUS examination of the colon. The recruited patients were examined by colon EUS with a radial-array echoendoscope [radial Scan Ultrasonic Video Endoscope EG-530UR2 (FUJIFILM Corporation, Tokyo, Japan) and an Ultrasound Processor SU-8000 (FUJIFILM Corporation, Tokyo, Japan)]. The risks and benefits of the procedure were described to the patients before the operation. The study protocol was approved by our university institutional review board as a retrospective study with prospectively entered database. The study was funded by King Chulalongkorn Memorial Hospital and faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Demographic data, including gender, age, and symptoms at presentation, were recorded. The endoscopic and EUS data, including the location of the lesion, the percent of circumferential involvement, the duration of the procedure, endoscopic findings, EUS findings, extent of tumor invasion, and the ability to pass the echoendoscope through the lesion, were also recorded. Ultrasonographic T staging was determined and recorded onsite by the endosonographer of the hospital (P.Kongkam). He was blinded to CT results prior to the procedure, as the results of the pre-procedural T stage would influence his judgments. The patients were sedated with conscious sedation (Meperidine and Midazolam) during procedures. Next, the patients were postoperatively observed in the recovery room according to the standard protocol for the endoscopy. After the procedure, the patients were followed up postoperatively by a physician on the team (S.L.). Any adverse events that arose during the procedure were noted. The clinical follow-up to detect any procedure-related adverse event was finished before any subsequent endoscopic or surgical removal. Within the next 4 wk, endoscopic or surgical resection was subsequently performed, and the surgical specimens were examined and pathologically T staged. The surgical pathological T stage was used as the gold standard against which the EUS T stage was compared. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy were also calculated for each pathological T stage.

### *Equipment and endoscopic technique*

The forward-viewing radial-array echoendoscope [radial Scan Ultrasonic Video Endoscope EG-530UR2 (FUJIF-

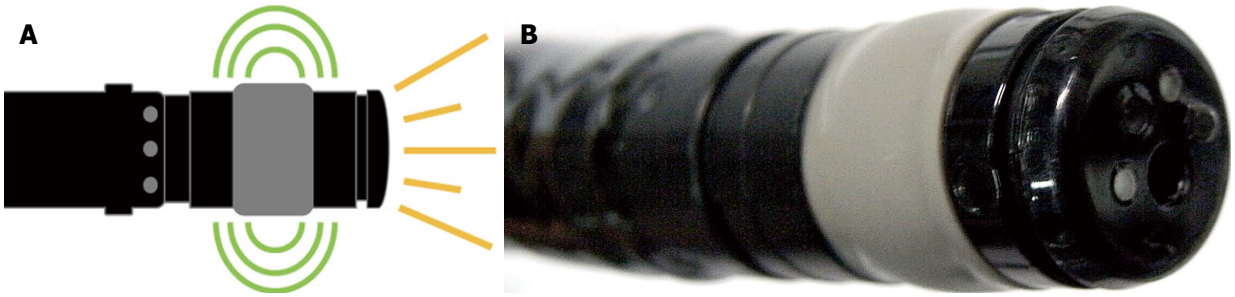


**Figure 1 Forward-viewing radial-array echoendoscope is a newly designed echoendoscope that provides a forward endoscopic view similar to that of a regular forward-viewing endoscope.** A: Echoendoscope provides forward endoscopic view (illustrated as yellow line), ultrasound wave is distributed perpendicularly to the tip of the echoendoscope as regular radial echoendoscope (illustrated as green line); B: Figures show the model of the forward-viewing radial-array echoendoscope (radial Scan Ultrasonic Video Endoscope EG-530UR2 (FUJIFILM Corporation, Tokyo, Japan).

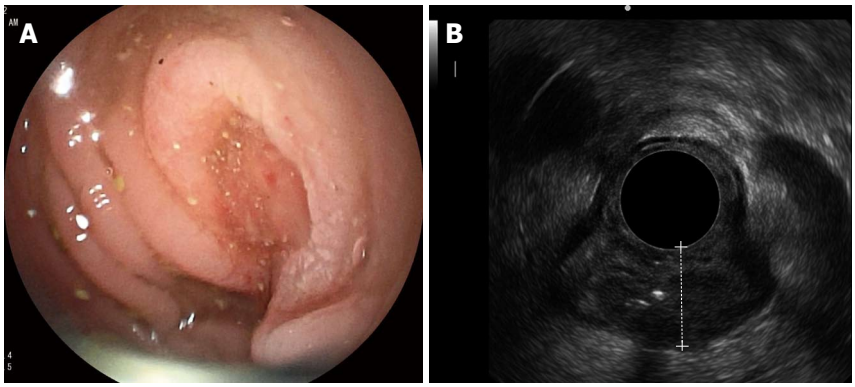


**Figure 2 Endoscopic and endosonographic images of colon cancer obtained from the novel forward-viewing radial-array echoendoscope (radial Scan Ultrasonic Video Endoscope EG-530UR2 (FUJIFILM Corporation, Tokyo, Japan) and Ultrasound Processor SU-8000 (FUJIFILM Corporation, Tokyo, Japan).** A: An endoscopic view of a colonic mass in sigmoid colon demonstrated by the echoendoscope; B: It demonstrated a hypo-echoic circumferential mass invading through muscularis propria layer (T3).

ILM Corporation, Tokyo, Japan) and Ultrasound Processor SU-8000 (FUJIFILM Corporation, Tokyo, Japan)] is a newly designed echoendoscope that provides a forward endoscopic view similar to that of a regular forward-viewing endoscope (Figure 1). Ultrasound waves were distributed perpendicularly to the tip of the echoendoscope, as in a regular radial echoendoscope. The frequencies included 5, 7.5, 10 and 12 MHz. In our study, we typically used 12 MHz for the staging of the tumor, as it was considered the optimal frequency to provide the best endosonographic imaging; however, the frequency was adjusted from 5 to 12 MHz to acquire the finest image. Theoretically, a lower frequency should provide farther endosonographic images, whereas a higher frequency should provide nearer images. All equipment was packaged in the format of a one-cart system. The echoendoscope had a small outer diameter of 11.4 mm with a forward endoscopic view of 140° and measured 120 cm in length.

Inserting the echoendoscope into the colon utilized the same maneuver as that used in the standard colonoscopy technique. However, because the echoendoscope was shorter than the regular colonoscope, the use of endoscopic techniques, such as pulling and shortening the scope, was encouraged. To illustrate the endosonographic images, water immersion was used to create an optimal endosonographic view for the staging of colon cancer. Water was not necessary if the endosonographic view was sufficient for staging. The balloon was not used to illustrate the endosonographic images. If the echoendoscope could pass through the lesions, it was placed in the middle of the lesion to most effectively stage the cancer. Nonetheless, if the echoendoscope could not be passed through the lesion, the stage was decided by the endosonographer at the location he deemed optimal. Figure 2A and B demonstrate the endoscopic and endosonographic images of malignant masses in the sigmoid colon. The lesion was endosonographically and pathologically staged as T3.

## RESULTS

During the study time, 82 patients with colon cancer located beyond rectum underwent colectomy. Twenty-one patients (25.6%) were recruited into the study. Eleven of them were male, and the mean age was 64.2 years (SD = 11.91), ranging from 43 to 85 years old. The presenting symptoms were abdominal pain ($n$ = 9), weight loss ($n$ = 8), hematochezia ($n$ = 3), melena ($n$ = 4), anemic symptoms ($n$ = 5), bowel habit changes ($n$ = 13), small caliber of stool ($n$ = 7), partial gut obstruction ($n$ = 4) and complete gut obstruction ($n$ = 2). A positive family

**Table 1 Number of patients with colon cancer classified according to endoscopic ultrasound *vs* surgical pathological T staging**

| EUS/Histology | T1 | T2 | T3 | T4 | Total |
|---|---|---|---|---|---|
| uT1 | 2 | 0 | 0 | 0 | 2 |
| uT2 | 0 | 3 | 2 | 0 | 5 |
| uT3 | 1 | 1 | 11 | 0 | 13 |
| uT4 | 0 | 0 | 0 | 1 | 1 |
| Total | 3 | 4 | 13 | 1 | 21 |

Tumors were pathologically staged as T1 ($n$ = 3), T2 ($n$ = 4), T3 ($n$ = 13) and T4 ($n$ = 1). Ultrasonographic T staging was T1 ($n$ = 2), T2 ($n$ = 5), T3 ($n$ = 13) and T4 ($n$ = 1). EUS: Endoscopic ultrasound; u: Ultrasound; T: Tumor.

history of colon cancer was found in 2 patients. Distant metastasis at the time of the EUS procedure was evident in 1 patient.

The mean time to reach the lesion was 3.52 min (SD = 2.09), and the mean procedural time was 7.10 min (SD = 1.41). The echoendoscope could pass through the lesions in 13 patients (61.9%). Among these, the echoendoscope reached the cecum in 10 patients (76.9%). The mean depth of the echoendoscope before reaching the lesions was 44.39 cm (SD = 27.21). No adverse events were found. The median duration from the date of EUS to surgery was 7 d (range from 1-40 d), and lesions were located in the cecum ($n$ = 2), ascending colon ($n$ = 1), transverse colon ($n$ = 2), descending colon ($n$ = 2), and sigmoid colon ($n$ = 14).

The tumors were pathologically staged as T1 ($n$ = 3), T2 ($n$ = 4), T3 ($n$ = 13) and T4 ($n$ = 1). The ultrasonographic T stagings were T1 ($n$ = 2), T2 ($n$ = 5), T3 ($n$ = 13) and T4 ($n$ = 1), as shown in Table 1. The overall accuracy rates of the echoendoscope for the T and N staging of colon cancer were 81.0% and 52.4%, respectively. The accuracy rates for T1, T2, T3 and T4 were 100%, 60.0%, 84.6% and 100%, respectively, as shown in Table 2. The accuracy rates among the traversable lesions ($n$ = 13) and obstructive lesions ($n$ = 8) were 61.5% and 100%, respectively. In comparison with other radiological imaging modalities, EUS and CT had overall accuracy rates of 81.0% and 68.4%, respectively (the data for CT were not available in 2 patients). The results are shown in Tables 2 and 3.

**Table 2 This table demonstrates the endoscopic ultrasound *vs* computed tomography staging of colon cancer in comparison with the surgical pathology classified into early (T1/T2) and advanced (T3/T4) stages**

| Pathological stage | T1/T2 | T3/T4 | Total |
|---|---|---|---|
| EUS ($n$ = 21) | 5 | 12 | 17 |
| CT ($n$ = 19) | 4 | 9 | 13 |

EUS and CT had overall accuracy rates of 81.1% and 68.4%, respectively. Data from CT was available in 19 patients. EUS: Endoscopic ultrasound; CT: Computed tomography.

**Table 3 Diagnostic values of endoscopic ultrasound *vs* computed tomography scan for staging of colon cancer**

| Modality | Sensitivity | Specificity | PPV | NPV | Accuracy |
|---|---|---|---|---|---|
| EUS ($n$ = 21 ) | 85.0% | - | 94.4% | - | 80.9% |
| CT scan ($n$ = 19) | 76.5% | - | 86.7% | - | 68.4% |

EUS: Endoscopic ultrasound; CT: Computed tomography; PPV: Positive predictive value; NPV: Negative predictive value.

## DISCUSSION

The current echoendoscope provides an oblique endoscopic view. This makes passing an echo-endoscope through the sigmoid colon impossible or significantly limited, as this part of colon is redundant and any scope with oblique viewing could not easily pass through it. Currently, only 3 types of echoendoscope can readily pass through the sigmoid colon: the miniprobe echoendoscope, the forward-viewing linear-array echoendoscope, and the forward-viewing radial-array echoendoscope, which was used in this study. While some studies use the first two types of echoendoscope to evaluate lesions in the colon beyond the rectum, this study utilized the forward-viewing radial-array echoendoscope, making us among the first in the world to assess its efficacy in evaluating and staging colon cancer in the colon far beyond the rectum. Bhutani *et al*[4] previously used a forward-oblique-viewing upper echoendoscope for T staging of sigmoid/left colon cancer and reported high accuracy rate. However, with the design of forward-oblique-viewing, it is not practical to pass it far beyond the rectum.

The physical properties of the first two echoendoscopes result in limitations in their capacity to stage colon cancer. For the miniprobe echoendoscope, the scope is so small that it can be inserted through the biopsy channel of the regular colonoscope. This allows the scope to pass through the entire colon. Therefore, this type of echoendoscope has been used to evaluate colon cancer in some studies[5]. A large German study prospectively recruited 50 patients with colonic tumors. Lesions were correctly classified in 17 adenomas: 16 T1, 8 T2, 5 T3 and 1 T4 cases of colon cancer. The total accuracy rate for T staging was 94%[6]. Considering the high accuracy rate of T staging with the miniprobe in colon cancer, this technique should be recommended as the tool of choice for staging colon cancer. However, in the German study, patients with locally advanced colon cancer were excluded, as the recruited patients were primarily referred for laparoscopic surgical removal. In addition, the majority of lesions were adenoma, not cancer. Therefore, the accuracy rate of 94% from the German study could not be directly compared with that in our study[6]. Another study from Germany of 88 patients with colon tumors reported an accuracy rate of 87%; however, similar to the prior study, 25 of the 88 patients had adenoma (T0), with an accuracy rate of 100%. Therefore, the reported accuracy rate once again

**Table 4 Advantages and disadvantages of the 3 types of echoendoscopes that have been used for the evaluation of lesions on the colon beyond the rectum**

| | Advantages | Disadvantages |
|---|---|---|
| A miniprobe echoendoscope | Widely available<br>Can be used together with regular colonoscope | Cannot be properly used for evaluation of thickened-wall colon cancer |
| A forward-viewing linear-array echoendoscope | Ability to perform EUS guided fine needle aspiration for colonic lesions | Inconvenient to evaluate circumferential colonic lesions like colon cancer |
| A forward-viewing radial-array echoendoscope | Ability to evaluate circumferential colonic lesions | Inability to perform EUS guided FNA for colonic lesions |

EUS: Endoscopic ultrasound.

could not be directly compared with that of the current study[7]. A small study using a miniprobe involving 17 and 13 patients with colon and rectal cancer, respectively, reported an accuracy of 70%[8]. Another study used the miniprobe to detect residual disease in malignant polyps, 12 of which were intra-mucosal and 9 of which were sub-mucosal. The results showed that the surgical pathology was free of cancer in 6 patients with normal endosonographic findings who underwent surgery[9]. In conclusion, the range of sound waves used by the miniprobe was too shallow to examine all of the layers of the colonic wall, particularly in advanced stages of cancer that infiltrate into the deeper colonic wall, leading to a much thicker colonic wall[10]. Therefore, it is considered unsuitable for colon cancer staging, particularly in cases of locally advanced colon cancer. In other cases, the results from past studies, including the above, showed that miniprobes, particularly those with high frequencies, are more suitable for the evaluation of early stages of colon cancer, classified as T1/T2[11-14].

The forward-viewing linear-array echoendoscope provides a front endoscopic view, allowing the echoendoscope to be readily passed to the cecum, according to the standard techniques for colonoscopy. However, because the sound wave was distributed in a linear direction, this technique is not suitable to evaluate circumferential lesions, such as colon cancer. In a feasibility study that used the forward-viewing linear-array echoendoscope in 15 patients with right side colonic sub-epithelial lesions, it was reported that the cecum was reached within 10 min in all patients. FNA was performed in 6 patients without any post-procedural adverse events[15]. This echoendoscope was then used to perform FNA from extra-colonic lesions. A study using a forward-oblique-viewing upper echoendoscope for the evaluation of 32 benign and malignant lesions reported an 85% accuracy rate for T staging in 20 patients with available surgical pathologies[4]. Another recent study reported data from the forward-viewing linear-array echoendoscope for an evaluation of 23 sub-epithelial lesions in the gastrointestinal tract. In 6 patients with colonic lesions, the echoendoscope could not reach the cecum in 1 patient[16]. This study, to our knowledge, is the first study in the world using the novel forward-viewing radial-array echoendoscope for colon cancer staging. The front endoscopic view makes the procedure's technique nearly the same as that used in standard colonoscopy, as it provides a similar endoscopic view, and the scopes have similar diameters. At present, a radial-array sound wave is suitable for the evaluation of circumferential lesions, as in colon cancer. Moreover, a wide range of wavelengths allows the echoendoscope to scan the extra-colonic area to search for any surrounding lymph node. The results from this study show that, in all patients, the lesions were reached without any adverse event. The time to reach the lesions was similar to that in standard colonoscopy techniques. This suggests that the forward-viewing radial-array echoendoscope [radial Scan Ultrasonic Video Endoscope EG-530UR2 (FUJIFILM Corporation, Tokyo, Japan)] and Ultrasound Processor SU-8000 (FUJIFILM Corporation, Tokyo, Japan) can be used safely as the staging tool of choice for colon cancer. The advantages and disadvantages of these 3 types of echoendoscope are compared and shown in Table 4.

The preoperative T staging of rectal cancer can be accurately performed by trans-rectal EUS and or MRI[10,17-20]. A recent meta-analysis of 42 studies ($n$ = 5039) using trans-rectal EUS for the staging of rectal cancer showed that the sensitivity and specificity rates of T1, T2, T3, and T4 were 87.8% and 98.3%, 80.5% and 95.6%, 96.4% and 90.6%, and 95.4 and 98.3%, respectively[21]. Based on the results of this meta-analysis, the accuracy rate of T2 tumors was the lowest. This was a common finding from the recruited studies in this meta-analysis. Similar trend was observed in our study. Our results suggested that the new type of EUS for the staging of colon cancers beyond the rectum provided an acceptable accuracy rate for T staging; however, it had a relatively low accuracy rate for N staging. Although surgical pathology was available for calculation in all of the cases in this series, the accuracy of this procedure from this study should not be consider conclusive, as the number of cases was too small. Furthermore, as the first study to use the echoendoscope to stage colon cancer, the learning curve could have reduced the accuracy of colon cancer staging. Future studies with a greater number of patients are thus required to definitively determine the accuracy of EUS for the T and N staging of colon cancer. The number of patients in future studies can be calculated based on the data from this study. As N staging significantly impacts the management of these cancers, further studies to clarify these answers are

strongly needed.

An accurate preoperative staging of rectal cancer by trans-rectal EUS influences definitive management. For example, T1/T2 rectal cancer can be managed with either endoscopic mucosal resection or endoscopic submucosal dissection, with a 5-year survival rate higher than 90%, whereas T3/T4 cancers should be removed with surgery[22-24]. For colon cancer, the data from the pilot phase of a recent randomized controlled trial (The FOxTROT trial) suggested that neoadjuvant therapy for locally advanced operable colon cancer significantly decreased TNM staging, compared with the postoperative group[25]. Therefore, if EUS was proven to be the most reliable tool for preoperative colon cancer staging, it should be combined with clinical practice before a decision is made to offer specific treatment to patients. CT data for the staging of colon cancer from a multi-center study in the UK showed that when CT was used for differentiation between early (T1/T2) and advanced stages of colon cancer, the sensitivity and specificity rates were 95% (95%CI: 87%-98%) and 50% (95%CI: 22%-77%), respectively[26]. The data from our study, despite the small number of patients, suggest that accuracy rate of EUS is clearly higher than that of CT scans, which are currently the tool of choice for staging colon cancer. In addition, with the current design of this new echoendoscope, it may be used in the future as a standard colonoscope for patients who have suspicious symptoms of colon cancer, as endoscopists can perform colonoscopy, tissue biopsy and endosonographic staging in the same procedure without the significant technical differences or adverse events from standard colonoscopy.

This study demonstrated that the forward-viewing radial-array echoendoscope is a feasible technique for staging colon cancer. The success and adverse event rates were low and similar to those of standard colonoscopy. However, systematic and larger studies with more patients must be conducted to specify the accuracy of the echoendoscope for this purpose.

## COMMENTS

### Background

Endoscopic ultrasound (EUS), rather than computed tomography (CT), was suggested as the staging tool of choice for rectal cancer as it demonstrates a higher accuracy of T and N staging compared to CT. Consequently, the authors speculated that if EUS can stage colon cancer beyond the rectum, it may yield more accurate results than CT and may improve the outcomes for colon cancer. Unfortunately, with the current design of EUS, it is not possible to use the EUS to stage colon cancer beyond the rectum.

### Research frontiers

The current echoendoscope provides an oblique endoscopic view. This makes passing an echo-endoscope through the sigmoid colon impossible, as this part of colon is redundant and any scope with oblique viewing could not pass through it. Currently, only 3 types of echoendoscope can pass through the sigmoid colon: the miniprobe echoendoscope, the forward-viewing linear-array echoendoscope, and the forward-viewing radial-array echoendoscope, which was used in this study. While some studies use the first two types of echoendoscope to evaluate lesions in the colon beyond the rectum, this study utilized the forward-viewing radial-array echoendoscope, making the authors among the first in the world to assess its efficacy in evaluating and staging colon cancer in the colon beyond the rectum. The physical properties of the first two echoendoscopes result in limitations in their capacity to stage colon cancer. For the miniprobe echoendoscope, the scope is so small that it can be inserted through the biopsy channel of the regular colonoscope. This allows the scope to pass through the entire colon. However, the range of sound waves used by the miniprobe was too shallow to examine all of the layers of the colonic wall, particularly in advanced stages of cancer that infiltrate into the deeper colonic wall, leading to a much thicker colonic wall. Therefore, it is considered unsuitable for colon cancer staging, particularly in cases of locally advanced colon cancer. The forward-viewing linear-array echoendoscope provides a front endoscopic view, allowing the echoendoscope to be readily passed to the cecum, according to the standard techniques for colonoscopy. However, because the sound wave was distributed in a linear direction, this technique is hence not suitable to evaluate circumferential lesions, such as colon cancer.

### Innovations and breakthroughs

The forward-viewing radial-array echoendoscope [radial Scan Ultrasonic Video Endoscope EG-530UR2 (FUJIFILM Corporation, Tokyo, Japan) and Ultrasound Processor SU-8000 (FUJIFILM Corporation, Tokyo, Japan)] is a newly designed echoendoscope that provides a forward endoscopic view similar to that of a regular forward-viewing endoscope. Ultrasound waves were distributed perpendicularly to the tip of the echoendoscope, as in a regular radial echoendoscope. All equipment was packaged in the format of a one-cart system. The echoendoscope had a small outer diameter of 11.4 mm with a forward endoscopic view of 140° and measured 120 cm in length. Inserting the echoendoscope into the colon utilized the same maneuver as that used in the standard colonoscopy technique. The novel forward-viewing radial-array echoendoscope can be readily passed through the colon.

### Applications

In this study, EUS was proven to be the most reliable tool for preoperative colon cancer staging. It hence should be combined with clinical practice before a decision is made to offer specific treatment to patients particularly in patients with locally advanced operable colon cancer.

### Peer review

Twenty-one colon cancers enrolled in 11 mo looks not really a relevant number. Authors should clarify their hospital volume (number of colectomies performed for cancer and number of standard endoscopies performed for cancer each year). Moreover, even though authors declared to have calculate the sensitivy, specificity, positive predictive value, negative predictive value of the EUS and of the standard radiology, the show exclusively the accuracy results.

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BRIEF ARTICLE

# Association of CD14/-260 polymorphism with gastric cancer risk in Highland Tibetans

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## Abstract

**AIM:** To investigate the relationship between CD14-260 and -651 polymorphisms and the risk of developing gastric cancer.

**METHODS:** DNA was extracted from peripheral blood samples obtained from 225 Tibetans with gastric cancer and 237 healthy Tibetans, and analyzed using the polymerase chain reaction/ligase detection (PCR/LDR) method to determine the genotypes at -260 and -651 loci of the CD14 promoter. The allele frequencies, genotype frequencies, and haplotypes were analyzed for their association with gastric cancer risk using online SHEsis software. The luciferase reporter assay and point mutation analysis were used to construct *in vitro* plasmids expressing a C/T homozygote at the -260 locus of the CD14 promoter.

**RESULTS:** The frequencies of CC, CT and TT genotypes in the CD14-260 C/T locus in gastric cancer patients were 19.1%, 38.7% and 42.2%, respectively, whereas they were 33.3%, 32.5% and 34.2%, respectively, in healthy control subjects. CT genotype carriers were more frequently found among gastric cancer patients than healthy controls (OR = 2.076; 95%CI: 1.282-3.360). Also, TT genotype carriers were more frequently found among gastric cancer patients (OR = 2.155; 95%CI: 1.340-3.466). Compared to the C allele of CD14/-260, the T allele was associated with an increased risk for gastric cancer (OR = 1.574; 95%CI: 1.121-2.045). Furthermore, the frequencies of CC, CT and TT in the CD14-651 C/T locus in gastric cancer patients were 64.4%, 29.3% and 6.2%, respectively, while they were 56.5%, 35.0% and 8.4%, respectively, in the healthy control subjects ($P > 0.05$). Data obtained using the luciferase reporter assay showed that the p260T homozygote was associated with greater CD14 promoter activity ($P < 0.01$).

**CONCLUSION:** CD14/-260 polymorphism is associated with gastric cancer risk in Highland Tibetans and affects CD14 promoter activity, thereby regulating CD14 expression.



**Key words:** CD14; Single nucleotide polymorphisms; Cancer susceptibility; Gastric cancer; Tibetans

**Core tip:** This is the first study to report that *CD14* gene polymorphisms in Highland Tibetans are associated with gastric cancer risk. We also identified a biomarker that can be used in gastric cancer research and clinical practice.

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## INTRODUCTION

Gastric cancer is a significant health problem and was the fourth most common malignancy worldwide in 2008, with an estimated 989600 incident cases. Approximately 72% of incident cases are diagnosed in developing countries; however, the incidence of gastric cancer in developed countries, including North America and Europe, has actually declined during the past 50 years[1]. The known risk factors for gastric cancer include *Heliobacter pylori* (*H. pylori*) infection, exposure to tobacco smoke, and high consumption of smoked foods, salted meat or fish, and pickled vegetables. Additionally, these risk factors augment the risk of gastric cancer in individuals who are genetically predisposed to the disease. However, consumption of fresh fruits and vegetables appears to lower the risk of gastric cancer[2-5]. Epidemiological studies have reported the presence of family clusters of gastric cancer and coincident cases of gastric cancer in identical twin siblings[6], suggesting that genetic susceptibility may impact the risk of developing gastric cancer. The genomic region of differentiation antigen 14 (CD14), especially the CD14 C→T polymorphisms in the -159/-260 and -651 promoter regions, has attracted significant attention regarding a possible role in gastric cancer. Such genetic polymorphisms might affect CD14 expression in cells and subsequently alter expression of genes downstream of CD14, thereby altering CD14 biological function and the development/outcome of CD14-related diseases[7,8].

CD14 is a glycoprotein located on the cell surface and functions as a receptor for lipopolysaccharides (LPSs). CD14 is mainly produced by monocytes, macrophages and neutrophils, and CD14-TLR4 is an important receptor complex in the LPS-presenting pathway[9]. *H. pylori* is a micro-aerophilic Gram-negative bacterium recognized by the World Health Organization (WHO) as a group I carcinogen for gastric cancer and lymphoma of gastric mucosa-associated lymphoid tissue. CD14 has multiple roles in the mediation of primary immune and inflammatory responses[10-12]. During an immune response, CD14 mediates cellular recognition of LPS, phosphorylation of cellular tyrosine, and translocation of nuclear factor (NF)-κB, to trigger release of cytokines and production of oxygen radicals. During an inflammatory response, CD14 functions in conjunction with LPS binding protein (LPB) to form a LPS-LPB-CD14-TLR4-MD2 complex, which then activates monocytes and macrophages to produce inflammatory cytokines.

To date, CD14-159 and/or -260 polymorphisms have been associated with susceptibility to or development of various diseases, including inflammatory bowel disease, allergies[13-16], and gastric cancer[17,18]. Tibetans have one of the highest prevalences of gastric cancer in China, and the prevalence in Tibet is higher than the average prevalence in China[19]. In this study, we used the ligase detection reaction (LDR) for nucleotide typing to examine the distribution of alleles and genotypes for the CD14 loci in Highland Tibetan gastric cancer patients, and compared the results with those obtained from healthy Highland Tibetans. This study was conducted to explore the association between CD14 polymorphisms and the risk of gastric cancer, and provide information regarding the molecular basis of gastric cancer risk in Highland Tibetans.

## MATERIALS AND METHODS

### Study subjects

A total of 225 gastric cancer patients and 237 healthy individuals were recruited from the Gastroenterology Unit and Oncology Unit of Tibet People's Hospital. This study was approved by the hospital's Institutional Review Board, and signed informed consent was obtained from all patients and healthy subjects before enrolling in the study. Gastric cancer was diagnosed based on criteria described by the WHO in 1979. Subjects with autoimmune disorders, including systemic lupus erythematous, rheumatoid arthritis, and inflammatory bowel disease, were excluded from this study. All study subjects were Highland Tibetans who had been living in Tibet for several generations and were not biologically related to one another.

### Cell line and culture

Gastric cancer cell line MGC-803 was obtained from the Shanghai Cell Bank of the Chinese Academy of Science (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin in a humidified incubator with 5% $CO_2$ at 37 ℃.

### Genomic DNA extraction and PCR amplification

Samples of venous blood (2 mL) were drawn *via* the cubital vein from fasting study subjects and stored in EDTA-containing anti-coagulative tubes at -70 ℃. DNA was extracted using a genomic DNA extraction kit (Beijing TIANGEN Biology Co., Ltd, Beijing, China) according to the manufacturer's protocol. CD14 gene polymorphisms were identified by searching GenBank for relevant CD14 sequence and polymorphism information. This information was then used to design PCR primers, which were synthesized and purified by Shanghai Sangon Biotech (Shanghai, China) (Table 1). PCR amplification was carried out in a 20 μL reaction volume containing 2.0 μL of 1 × PCR buffer, 0.4 μL of each primer (10 pmol), 2.0 μL of each dNTP (2.0 mmol/L), 9.3 μL of sterilized water, 0.6 μL of $MgCl_2$, 0.3 μL of *Taq* enzyme (2.5 U/μL), and 5 μL of template DNA. The PCR reaction conditions consisted of an initial denaturation at 95 ℃ for 15 min, followed by 35 cycles of 94 ℃ for 30 s, 53 ℃

**Table 1 Primers and probes used for genotyping CD14-260C/T and -651C/T**

| Polymorphism | Primers and probes | Length, bp |
|---|---|---|
| -260 C/T | 5'-CACCCACCAGAGAAGGCTTA-3' | 212 |
| | 5'-ATCACCTCCCCACCTCTCTT-3' | |
| Common probe | 5'-CCCCCTCCCTGAAACATCCTTTTTTTTTTTTTTTTTT-FAM-3' | |
| Discriminating probes | 260C/T-R_G | 77 |
| | 5'-TTTTTTTTTTTTTTTTTGCAGAATCCTTCCTGTTACGGC | |
| | 260C/T-R_A-3' | 79 |
| | 5'-TTTTTTTTTTTTTTTTTTGCAGAATCCTTCCTGTTACGGT-3' | |
| -651C/T | 5'-GGGTAGAATTAGGTTCAAG-3' | 103 |
| | 5'-CTTAATCAAAGGAGCAAGG-3' | 1 |
| Common probe | 5'-GTCTAAAGAAAAATTCCCCCTTCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-FAM-3' | |
| Discriminating probes | 651C/T_C | 150 |
| | 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAGGTTCAAGAAAAGGAAGTTG-3' | |
| | 651C/T_T | 152 |
| | 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAGGTTCAAGAAAAGGAAGTTA-3' | |

for 1 min and 65 ℃ for 1 min, and a final extension cycle at 72 ℃ for 7 min. The PCR products were separated by 3% agarose gel electrophoresis and used for the LDR.

### LDR

The LDR was used to measure the distribution of CD14 alleles and genotypes in the aforementioned PCR products. The upstream and downstream probes for multiple LDRs were designed as shown in Table 1. The upstream probe was modified by phosphorylation at the 5'-terminal region. The LDR reaction mixture contained 2 μL of PCR product, 1 μL of LDR probe mixture (1 mmol/L), 1 μL of 1 × buffer, and 0.05 μL of ligase (2 U), and the total volume was adjusted to 10 μL using PCR-grade water. LDR conditions were 95 ℃ for 2 min, followed by 30 cycles of 95 ℃ for 15 s and 50 ℃ for 25 s. The PCR/LDR gel electrophoresis and DNA sequencing experiments were conducted by Shanghai Biowing Applied Biotechnology Co., Ltd. (Shanghai, China) using the following equipment: Gene Amp PCR System 9600 (Perkin-Elmer, Waltham, MA, United States); PTC-200 Gradient Cycler (MJ Research, Waltham, MA, United States); PRISM 3730 and 3100 DNA Sequencer (Applied Biosystems Inc., Carlsbad, CA, United States); JY600+ Electrophoresis System (Beijing Junyi-Dongfang Electrophoresis Equipment Co., Ltd, Beijing, China); FR-200A Automatic Ultra-violet and Visible Light Analyzer and the Biological Electrophoretic Image Analyzer (Shanghai Furi Technology Co., Ltd, Shanghai, China); Agarose LE (Shanghai Genebase Gene-Tech Co., Ltd, Shanghai, China); Flouroskan Ascent FL (Thermo Scientific, Waltham, MA, United States).

### Construction of reporter vectors

The luciferase reporter assay was used to examine the effects of different genotypes (C and T) of CD14 on regulation of CD14 expression in gastric cancer cells. Reporter vectors were constructed to verify the effect of -260C/T polymorphism on CD14 expression, and primers were used to amplify the -360 to +29 region of the CD14 promoter. The relevant primer sequences were: 5'-GACCGCTAGCCGAGTCAACAGGGCATTCAC-3' and 5'-CGTCAAGCTTGTT CGACCCCAAGACCCTAC-3', while primers for the mutated-260 site (changed to C) were 5'-CCTTCCTGTTACGGCCCCCCTCCCTG-3' and 5'-GTTTCAGGGAGGGGGGCCGTAACAGGA-3'. Cleavage sites of *Nhe* I and *Hin*dⅢ were also introduced on both ends of the primers, respectively. The PCR products were inserted into a pGL3-Basic reporter vector (Promega, Madison, WI, United States). After cloning, amplification, and DNA sequence confirmation, these vectors were designated as p260C and p260T, respectively, and subsequently used to transfect gastric cancer MGC-803 cells. Co-transfection with the renilla luciferase reporter vector pRL-TK was also performed at this time. After 12 h, LPS was added to the cell culture at a final concentration of 1 μg/mL, and the cells were incubated for an additional 12 h and then analyzed using a dual-luciferase assay kit (Promega) according to the manufacturer's instructions. Measurements of fluorescence intensity were expressed as the mean ± SE of firefly/renilla obtained from three readings at each setting.

### Statistical analysis

Frequencies of CD14 genotypes and alleles in gastric cancer patients and control subjects were calculated and tested using the Hardy-Weinberg equilibrium equation. Allele frequencies, genotype frequencies, and haplotypes were analyzed using SHEsis online software (http://analysis.bio.-x.cn/myAnalysis.php). Comparisons of demographic and clinical data between groups were conducted using the $\chi^2$ test and Student's *t* test. The Student's *t* test was also used to analyze genotype and luciferase levels. *P* values were generated using the SPSS statistical software suite for Windows (SPSS Inc., Chicago, IL, United States); a *P*-value < 0.05 was considered statistically significant.

**Table 2 Characteristics of study subjects *n* (%)**

| Variable | Controls *n* = 237 | GC patients *n* = 225 | [a]*P* value |
|---|---|---|---|
| Age, yr | 54.8 ± 11.2 | 54.0 ± 12.3 | NS |
| Male | 175 (73.8) | 172 (76.4) | NS |
| Clinical stage | | | |
| I | | 7 (3.1) | |
| II | | 21 (9.3) | |
| III | | 126 (56.0) | |
| IV | | 71 (31.6) | |

[a]GC *vs* controls by Student's *t* test or $\chi^2$ test. GC: Gastric cancer; NS: Not significant.

## RESULTS

### Characterization of gastric cancer and control subjects

Genotyping for the CD14 polymorphisms was conducted in 225 patients pathologically diagnosed with gastric adenocarcinoma and 237 healthy control subjects. The gastric cancer patients had a mean age of 54.8 ± 11.2 years, and included 172 males and 53 females. The 237 healthy control subjects were recruited among individuals who received a routine health examination at the hospital during the concurrent period. The control subjects had a mean age of 54.8 ± 11.2 years, and included 175 males and 62 females (Table 2). The results of genotyping studies showed that genotype frequencies of CD14 -260C/T and -651 C/T were not significantly different ($P > 0.05$) between gastric cancer and control subjects. The population was in Hardy-Weinberg equilibrium.

### Distribution of genotypes and alleles of CD14-260C/T

Gastric cancer patients showed CD14-260 C/T CC, CT and TT genotype frequencies of 19.1%, 38.7% and 42.2%, respectively, whereas these frequencies in healthy control subjects were 33.3%, 32.5% and 34.2%, respectively. These results showed that frequencies of the CT and TT genotypes of CD14 were significantly higher in gastric cancer patients than in healthy control subjects (for CT: OR = 2.076; 95%CI: 1.282-3.360, $P = 0.003$; for TT: OR = 2.155; 95%CI: 1.340-3.466, $P = 0.001$). Additionally, the T allele carried by gastric cancer patients was associated with a diagnosis of gastric cancer (OR = 1.574; 95%CI: 1.121-2.045, $P = 0.001$) (Table 3). The DNA sequences of CD14-260C/T polymorphism are shown in Figure 1A.

### Distribution of genotypes and alleles of CD14-651 C/T

The frequencies of CD14-651C/T CC, CT and TT genotypes in gastric cancer patients were 64.4%, 29.3% and 6.2%, respectively, and 56.5%, 35.0% and 8.4%, respectively, in the healthy control subjects. The differences in genotype frequencies between the two groups of subjects were not statistically significant ($P > 0.05$). Moreover, the frequencies of C and T alleles were 79.1% and 20.9%, respectively, in gastric cancer patients, and 74.1% and 25.9%, respectively, in healthy control subjects, and these differences were also not statistically significant ($P > 0.05$; Table 3). The DNA sequences of CD14-651C/T polymorphism are shown in Figure 1B.

**Table 3 Genotype and allele frequencies ofCD14-260C/T and -651C/T in gastric cancer patients and healthy control subjects *n* (%)**

| Polymorphism | Case *n* = 225 | Control *n* = 237 | OR | 95%CI | *P* value |
|---|---|---|---|---|---|
| -260 C/T | | | | | |
| Genotypes | | | | | |
| CC | 43 (19.1) | 79 (33.3) | 1.000 | (reference) | - |
| CT | 87 (38.7) | 77 (32.5) | 2.076 | 1.282-3.360 | 0.003 |
| TT | 95 (42.2) | 81 (34.2) | 2.155 | 1.340-3.466 | 0.001 |
| Alleles | | | | | |
| C | 173 (38.4) | 235 (49.6) | 1.000 | (reference) | - |
| T | 277 (61.6) | 239 (50.4) | 1.574 | 1.121-2.045 | 0.001 |
| -651 C/T | | | | | |
| Genotypes | | | | | |
| CC | 145 (64.4) | 134 (56.5) | 1.000 | (reference) | - |
| CT | 66 (29.3) | 83 (35.0) | 0.735 | 0.493-1.096 | 0.130 |
| TT | 14 (6.2) | 20 (8.4) | 0.647 | 0.314-1.332 | 0.234 |
| Alleles | | | | | |
| C | 356 (79.1) | 351 (74.1) | 1.000 | (reference) | - |
| T | 94 (20.9) | 123 (25.9) | 0.753 | 0.55-1.024 | 0.07 |

### Haplotype analysis of CD14-260C/T and -651 C/T loci

Linkage disequilibrium (LD) analysis showed linkage disequilibrium between the CD14/-260C/T and CD14/-651 C/T loci (|D'| > 0.50). Haplotype analysis showed that the -260C/T--651 C/TT-C haplotype was associated with an increased risk of developing gastric cancer (OR = 1.58; 95%CI: 1.21-2.07, $P = 0.001$; Table 3), and the -260C/T--651 C/TC-C haplotype was associated with a decreased risk of developing gastric cancer (OR = 0.70; 95%CI: 0.51-0.96, $P = 0.028$; Table 4).

### Effect of -260C/T gene promoter on CD14 expression

The luciferase reporter assay was used to determine the effect of the CD14-260C/T gene promoter on CD14 expression. The results showed that luciferase activity in p260T-transfected cells was 2.05-fold higher than that in p260C-transfected cells ($P < 0.01$). LPS treatment induced 5.19- and 6.09-fold increases in relative luciferase activity in p260C- and p260T-transfected cells, respectively ($P < 0.01$). The 1.54-fold increase in relative luciferase activity produced by LPS treatment in p260T-transfected cells compared to p260C-transfected cells ($P < 0.01$) indicated that the CD14-260 polymorphism did affect CD14 expression in gastric cancer cells (Figure 2).

## DISCUSSION

This study analyzed polymorphisms of CD14/-260 and CD14/-651 loci in Tibetan gastric cancer patients and healthy control subjects. The results showed that compared to the C allele, the T allele of CD14/-260 was associated with an increased risk for gastric cancer. However, the CD14/-651 polymorphism was not associated

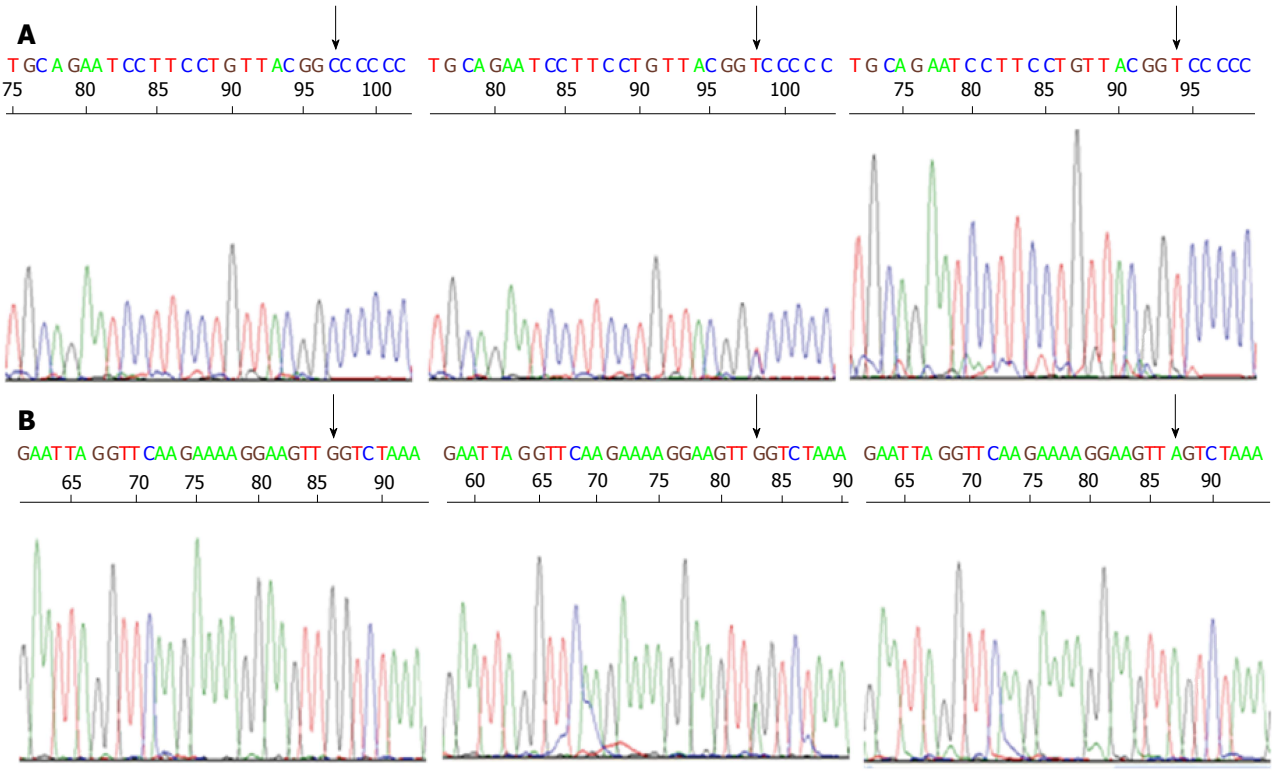


**Figure 1 DNA sequences of CD14-260C/T and CD14-651C/T polymorphisms.** A: CD14-260C/T polymorphism; B: CD14-651C/T polymorphism.

**Table 4 Haplotype distribution of CD14-260C/T and -651 C/T in gastric cancer patients and healthy control subjects *n* (%)**

| Haplotypes -260C/T - -651 C/T | Case | Control | OR (95%CI) | *P* value |
|---|---|---|---|---|
| T-C | 244 (57.9) | 206 (46.5) | 1.58 (1.21-2.07) | 0.001 |
| C-C | 87 (20.8) | 120 (27.2) | 0.70 (0.51-0.96) | 0.028 |
| C-T | 76 (18.1) | 97 (21.9) | 0.79 (0.56-1.10) | 0.157 |
| T-T | 13 (3.3) | 19 (4.4) | 0.73 (0.36-1.47) | 0.371 |

Global $\chi^2$ is 11.44, degrees of freedom = 3, $P$ = 0.009. Bolded values are statistically significant.

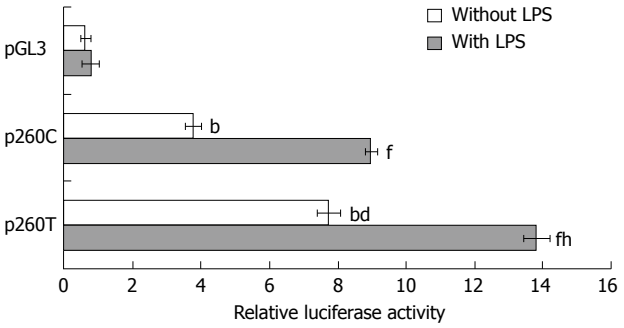


**Figure 2 Effect of CD14-260C/T polymorphism and lipopolysaccharide treatment on CD14 expression.** Luciferase reporter vectors carrying the CD14-260C/T polymorphism and renilla luciferase reporter vector pRL-TK were co-transfected into gastric cancer MGC-803 cells and treated with 1 μg/mL of LPS. Later, cells were lysed and assayed for luciferase activity. Data are normalized to the mean ± SE value of Firefly/Renilla. [b]$P$ < 0.01 *vs* control cells without LPS; [d]$P$ < 0.01 *vs* p260C-transfected cells without LPS treatment; [f]$P$ < 0.01 vs control cells with LPS treatment; [h]$P$ < 0.01 *vs* p260C-transfected cells with LPS treatment. LPS: Lipopolysaccharide.

with a risk of gastric cancer. Moreover, the CD14/-260 polymorphism was found to affect CD14 promoter activity and therefore regulate CD14 expression. Our current study confirmed that the CD14/-260 polymorphism is associated with a higher risk for developing gastric cancer in Highland Tibetans. A further study with a larger number of subjects will be conducted to investigate the molecular mechanism underlying the role of CD14 in development of gastric cancer.

Gastric cancer is associated with *H. pylori* infection and subsequent inflammation, and thus the role of cytokines in gastric cancer has received increased attention. Because environment, lifestyle, and other extrinsic factors also impact gastric cancer development, research on gene-environment interactions may provide a better understanding of the genetic background of various ethnic groups and why certain ethnic groups have increased susceptibility to developing gastric cancer.

Various epidemiological studies have shown inconsistent correlations between different diseases and CD14 polymorphisms, and some of these contradictory results might be due to the inclusion of heterogeneic genetic groups in such studies. Highland Tibetans live in a hypoxic environment and have a unique genetic makeup that permits adaptation to such environmental conditions. Highland Tibetans also frequently suffer from certain pre-cancerous conditions, such as chronic atrophic gastritis and gastric ulcer. These conditions may be due to high levels of circulating *H. pylori*, which are associated with the high prevalence of gastric cancer in

Tibetans[20]. The current study demonstrated a higher rate of CD14/-260 CT genotype polymorphism in gastric cancer patients (38.7%) than in healthy control subjects (32.5%). Additionally, the CD14/-260 TT genotype was found more frequently in gastric cancer patients (42.2%) compared to healthy subjects (32.2%). The T allele of CD14/-260 was also found significantly more often in gastric cancer patients than in healthy subjects, and individuals carrying the T allele had a 1.6-fold increased risk of developing gastric cancer compared to individuals carrying the C allele. Highland Tibetans carrying the T allele of CD14/-260 might have an increased risk of gastric cancer, because the T allele promotes high levels of CD14 expression. CD14/-651 C/T polymorphism was not associated with a risk for gastric cancer in Highland Tibetans. Our current data support results from a previous study by Zhao *et al*[18], which showed an association between genetic polymorphism in this locus and *H. pylori*-related gastric cancer.

As a receptor for LPS, CD14 plays a crucial role in innate immunity and is mainly expressed in mature monocytes, macrophages, and activated neutrophils[21]. Inflammatory signals induced by LPS initiate signal transduction through TLR4 and CD14. These events trigger activation of transcription factors, such as NF-κB, which regulate secretion of interleukins-1, 6, 8 and 12, and TNF-α. The latter cytokine subsequently triggers a series of immune and inflammatory responses that can damage the gastric mucosa[22]. However, the mechanism by which *H. pylori* infection causes gastric cancer remains to be determined. One hypothesis is that inflammation triggered by *H. pylori* infection causes the gastric mucosa to undergo atrophy, intestinal metaplasia, and dysplasia, leading to eventual development of gastric cancer[23]. In this context, polymorphism of CD14/-260 may alter the host's immune system, weaken defenses against *H. pylori* infection, and allow the gastric mucosa to become susceptible to infection, inflammation, and formation of cancerous lesions. At the molecular level, the C260T polymorphism harbors the S1 binding site of the CD14 promoter, and a C/T polymorphism may alter CD14 promoter activity, leading to increased CD14 gene transcription. Thus, the T allele homozygote can enhance CD14 expression on circulating monocytes and therefore promote inflammation. Our current data support this notion, because CD14/C260T was associated with very high luciferase activity, and such high transcriptional activity will induce high levels of CD14 expression, especially during *H. pylori* infection[18].

Gastric cancer is a multi-factorial disease, and our current study suggests that gastric cancer may be induced by several events, including oncogene activation and the down-regulation of tumor suppressor genes. Nevertheless, this study does not provide evidence associating CD14 polymorphisms with these events or explain how CD14 polymorphisms subvert the immune response to trigger gastric cancer development. However, our study does provide novel information which may link CD14 polymorphisms in Highland Tibetans with an increased risk of developing gastric cancer. Future studies will further investigate the role of CD14 in development of gastric cancer.

## COMMENTS

### Background

*Helicobacter pylori* infection is the major risk factor for gastric cancer. During an immune response, CD14 mediates cellular recognition of lipopolysaccharides (LPS), phosphorylation of cellular tyrosine, and translocation of nuclear factor (NF)-κB to trigger cytokine release and production of oxygen radicals. This study investigated CD14-260 and -651 polymorphisms in Highland Tibetans for their association with gastric cancer risk.

### Research frontiers

Tibetans have one of the highest rates of gastric cancer in China, and the prevalence in Tibet is higher than the average prevalence throughout China. This study was conducted to explore the association between CD14 polymorphisms in highland Tibetans and the risk of gastric cancer, and also to clarify the connection between genotype and phenotype.

### Innovations and breakthroughs

This study demonstrated an association between CD14/-260 polymorphism and gastric cancer risk in Highland Tibetans. Studies conducted *in vitro* revealed that CD14/-260 polymorphism affects CD14 promoter activity and may therefore regulate CD14 expression. This study provides information regarding the molecular basis for an increased gastric cancer risk among Highland Tibetans.

### Applications

This study provides molecular data linking genetic polymorphisms to an increased risk of gastric cancer in the high-plateau Tibetan population in China. The results lay a foundation for use of genetic screening to identify individuals at high risk of gastric cancer and for developing gene therapy techniques for prevention and treatment.

### Terminology

LPS is lipopolysaccharide. CD14 is a cell surface glycoprotein mainly produced by monocytes, macrophages, and neutrophils. CD14 has multiple roles in the mediation of primary immune and inflammatory responses. CD14-TLR4 is an important receptor complex in the LPS presenting pathway.

### Peer review

This is a well designed study with interesting results. The authors demonstrated an association of CD/-260 polymorphism with gastric cancer risk in Highland Tibetans. *In vitro* data revealed that CD14/-260 polymorphism affects CD14 promoter activity and therefore regulates CD14 expression. This manuscript has some novelty because the study population was mainly located in Tibet, and the results may help explain the high prevalence of gastric cancer in this highland area.

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META-ANALYSIS

# Antiviral therapy in cytomegalovirus-positive ulcerative colitis: A systematic review and meta-analysis

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## Abstract

**AIM:** To evaluate the impact of antiviral treatment on cytomegalovirus (CMV)-positive ulcerative colitis patients.

**METHODS:** We performed a systematic review and meta-analysis (MA) of comparative cohort and case-control studies published between January 1966 and March 2013. Studies focusing on colectomy series and studies including only less than 3 patients in the treated or non-treated arm were excluded. The primary outcome was colectomy within 30 d of diagnosis. Secondary outcomes included colectomy during the follow-up period Subgroup analyses by method of detection of CMV, study design, risk of bias and country of origin were performed. Quality of studies was evaluated according to modified New-Castle Ottawa Scale.

**RESULTS:** After full-text review, nine studies with a total of 176 patients were included in our MA. All the included studies were of low to moderate quality. Patients who have received antiviral treatment had a higher risk of 30-d colectomy (OR = 2.40; 95%CI: 1.05-5.50; $I^2$ = 37.2%). A subgroup analysis including only patients in whom CMV diagnosis was based did not demonstrate a significant difference between the groups (OR = 3.41; 95%CI: 0.39-29.83; $I^2$ = 56.9%). Analysis of long-term colectomy rates was possible for 6 studies including 110 patients. No statistically significant difference was found between the treated and untreated groups (OR = 1.71; 95%CI: 0.71-4.13; 6 studies, $I^2$ = 0%). Analysis of mortality rate was not possible due to a very limited number of cases. Stratification of the outcomes by disease severity was not possible.

**CONCLUSION:** No positive association between antiviral treatment and a favorable outcome was demonstrated. These findings should be interpreted cautiously due to primary studies' quality and potential biases.



**Key words:** Ulcerative colitis; Cytomegalovirus; Colectomy; Antiviral treatment; Gancyclovir; Foscarnet

**Core tip:** We have undertaken a meta-analysis of the existing literature in order to evaluate the impact of antiviral therapy on the outcome (colectomy rate) of ulcerative colitis patients with documented presence of cytomegalovirus. Nine studies of low to moderate quality with significant heterogeneity were included. Patients treated with antivirals did not have a better outcome in comparison to those who were not. These results should be interpreted with caution in view of low quality of the included studies and several potential biases. Additional high-quality studies are required to define the optimal diagnostic and therapeutic strategy for these patients.

Kopylov U, Eliakim-Raz N, Szilagy A, Seidman E, Ben-Horin S, Katz L. Antiviral therapy in cytomegalovirus-positive ulcerative colitis: A systematic review and meta-analysis. *World J Gastroenterol* 2014; 20(10): 2695-2703 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i10/2695.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i10.2695

## INTRODUCTION

Cytomegalovirus (CMV) infections are very common in the general population[1]. The infection is usually clinically mild and often confused with other minor viral infections. Reported rates of CMV-IgG positivity, signifying past exposure to the virus, are as high as 100%, depending on the age and geographic location of the population studied[2]. Even though systemic CMV disease in the immunocompetent patients is extremely rare, primary CMV infection or reactivation may lead to disseminated disease or end-organ involvement in immunocompromised patients (post-solid organ transplantation, chemotherapy-treated, HIV, recipients of immunosuppressive drugs, *etc.*)[3]. CMV is the most common virus causing disease and death in solid organ transplant recipients (kidney, heart, liver, lung and pancreas)[4].

The role of CMV infection in patients with inflammatory bowel disease (IBD) is controversial. Although CMV has been specifically associated with refractory disease, the strength and nature of this association has been a subject of debate[5]. The detection of CMV in the colon is frequent in patients with acute severe ulcerative colitis (UC). CMV has been reported to be present in colonic tissue of 21%-34% of all UC patients and in 33%-36% of steroid refractory patients[6].

The clinical significance of detecting CMV in UC remains debatable[7]. It has been suggested that intestinal CMV detection may be a marker of severe disease that is more likely to be refractory to corticosteroid and immunosuppressive therapy. Alternatively, CMV might only be an "innocent bystander", reflecting a remote infection of the involved mucosa, without significantly impacting on outcomes[2]. Conversely, CMV is often considered an undisputable infection with potentially grave outcome. As such, the standard therapy for CMV infection in UC patients includes intravenous gancyclovir, with a possible switch to oral valgancyclovir upon improvement. Intravenous foscarnet is usually reserved for patients who do not tolerate or respond to gancyclovir[2]. However, prospective controlled trials to validate the clinical benefit of such treatment in IBD patients are still largely unavailable.

The aim of this study is to evaluate the impact of antiviral therapy on the outcome of CMV-positive UC patients using a systematic review and meta-analysis (MA) of the published studies pertaining to the subject.

## MATERIALS AND METHODS

### Search strategy

We searched Pubmed, Embase and World of Science databases for articles published between 1966 and March 2013. In addition, we manually scanned the abstracts presented at the following medical conferences: DDW, UEGW, ECCO for the years 2004-2012. In our analysis, we have included studies published as full papers or conference abstracts.

The search strategy included the following search terms: "ulcerative colitis" or "inflammatory bowel disease" and "cytomegalovirus" or "CMV". Their MESH terms were crossed. We manually scanned references of all included studies to identify additional relevant publications.

### Study selection

We included prospective and retrospective cohort studies comparing outcomes of treated and untreated CMV-positive UC patients.

The following types of studies were excluded: (1) Studies including less than 4 patients in one or more of the arms (treated and non treated patients); (2) Studies pertaining exclusively to CD patients. For studies involving mixed cohorts (CD and UC), we analyzed only the UC data. If this was not possible, we contacted the first and last author of the study and requested that they provide the relevant data. If no response was received, the study was excluded from analysis; (3) Studies describing antiviral treatment with an antiviral agent other than gancyclovir, valgancyclovir or foscarnet were also excluded; and (4) Series exclusively reporting colectomy data, *i.e.* only patients who reached the outcome of colectomy, were included. Patients were excluded from the analysis if they underwent colectomy before gancyclovir treatment was considered or the results of CMV assessment were available.

### Outcome measures

The primary outcome assessed was the rate of colectomy during the hospitalization period or within 30 d of diagnosis. Secondary outcomes was colectomy rate for the available follow-up duration (3 mo since the index hospitalization).

Subgroup analysis was done according to the method of CMV diagnosis, study design, study location and quality of studies, based on Newcastle Ottawa Scale (see below).

Data extraction and quality of data assessment: Two reviewers (UK, NE) independently applied inclusion criteria, selected the studies, and extracted data, outcomes and quality. In cases with disagreement between the two reviewers, the issues were resolved by discussion. Authors of studies were contacted if clarification was needed. The following data were collected: period and location of the studies, year of publication, inclusion criteria for participants in each study, demographic and disease characteristics of the included patients, method of diagnosis of CMV colitis, details of anti-inflammatory treatment preceding the diagnosis of CMV, type, duration and dose of the antiviral treatment, and outcome measures as mentioned above.

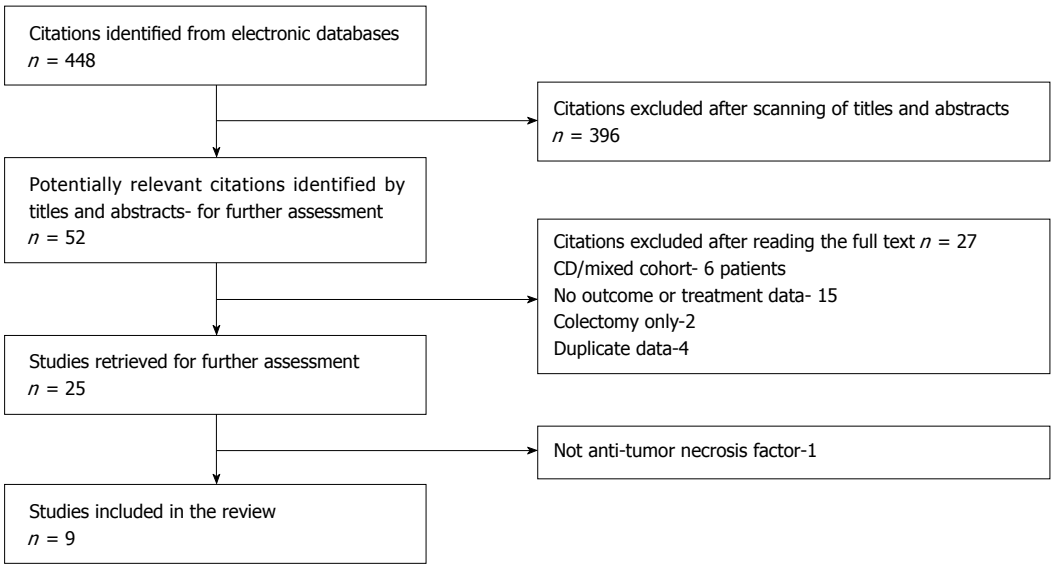



**Figure 1 Selection of studies on cytomegalovirus infection complicating ulcerative colitis.**

Quality of studies was assessed using the Newcastle Ottawa Scale (NOS), modified for this review. The quality of the included studies was evaluated based on questions regarding the selection and comparability of the cohort (treated and non-treated CMV-infected UC patients) and the outcome of the study. A higher score indicates higher methodological quality. We defined high-quality studies as mean total points ≥ 5; low quality as < 4, and moderate, between those values.

### *Statistical analysis*

Study results were expressed as odds ratio (OR) with 95%CI. We used a fixed-effect model to pool results. We assessed heterogeneity using the $\chi^2$-test of heterogeneity and the $I^2$ measure of inconsistency. If significant heterogeneity had a $\chi^2$-test $P$ value < 0.1 or an $I^2$ measure > 50% we conducted a random effects meta-analysis. Sensitivity analysis was done without the less qualified studies or the study which showed the most significant difference, with the same statistical methods. Analyses were performed using RevMan 4.2 (The Nordic Cochrane Centre, The Cochrane Collaboration Copenhagen, 2003).

## RESULTS

Our search yielded 448 studies. After title and abstract scanning 397 of them were excluded. An additional 42 studies were excluded after full-text review (Figure 1). A total of 9 studies[8-16] were included in our systematic review and MA, with data available for 176 patients. Table 1 shows the characteristics of the included studies.

All studies collected data on patients admitted between 1990-2011 and published between 2010 and 2013. Four studies were conducted in Europe[9,10,13,17], two in Japan[8,15] and the others in Canada[14], South Korea[12] and Israel[16]. Six studies were published as ull text articles[8,12-14,16,17] and three[9,10,15] as conference abstracts. Duration of follow-up ranged between 1 to 40 mo. Four studies[8,12,13,17] were prospective and five[9,10,14-16] retrospective. In four studies, the primary objective was to compare the outcome of patients treated with antivirals to the outcome of patients who did not receive antiviral therapy[9,12,15,16]. The quality of the included studies was determined according to the modified Newcastle Ottawa Scale (Table 2). The overall quality of the studies was moderate-low; no study met the criteria for high quality.

### *Outcomes*

Analysis of colectomy rates during hospitalization was possible for all included studies[8-16] (Figure 2) and for all patients (70 treated, 106 controls). Patients who had received antiviral treatment had a higher risk of requiring a subsequent colectomy (OR = 2.40; 95%CI: 1.05-5.50; $I^2$ = 37.2%). Subgroup analysis including only patients in whom CMV diagnosis was based on immunohistochemistry (IHC) staining showed the same trend, with much wider CI (OR = 3.41; 95%CI: 0. 39-29.83; 5 studies, Random effect, $I^2$ = 56.9%). Subgroup analyses using only the prospective studies ($n$ = 4) or studies with moderate ($n$ = 3) *vs* high risk of bias ($n$ = 6) showed the same trend, but without reaching statistical significance because of small group size and wide CI. We performed an additional subgroup analysis, comparing studies conducted in Europe[9,10,13,17] to those taken place in Asia[8,12,15] (omitting the Canadian and Israeli studies). No case of colectomy during hospitalization was reported among the non treated group in the Asian studies, therefore, patients who received antivirals underwent more colectomies than the non treated patients in the Asian studies. In the European studies no difference was recorded between the two groups (OR = 19.85; 95%CI: 1.94-203.61; and OR = 0.81; 95%CI: 0.24-2.79 for studies taken in Asia and in Europe, respectively).

**Table 1 Characteristics of the included studies and patients on cytomegalovirus in ulcerative colitis**

| Ref. | Year of publication | Type | Design | *n* | | Included patients | Severity criteria | Method of diagnosis | Anti-viral tx | Follow-up (mo) | Short-term colectomy rate | | Long-term colectomy rate | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | TX | C | | | | | | TX | C | TX | C |
| Omiya *et al*[8] | 2010 | Full | P[1] | 10 | 10 | Moderate to severe UC | Seo's score | Tissue PCR (IHC/HE -) | GCV | 12 | 0/10 | 0/10 | 3/10 | 0/10 |
| Zeki *et al*[9][1] | 2010 | Abstract | R[2] | 7 | 10 | UC (no severity data) | NA | HE/IHC+ | GCV-5, VGCV-2 | 12 | 4/7 | 7/10 | 4/7 | 7/10 |
| Maconi *et al*[10][3] | 2011 | Abstract | R[2] | 6 | 14 | Moderate to severe UC | Mayo/ Baron score | HE/IHC+ | GCV | 12 | 0/6 | 0/14 | 0/6 | 2/13 |
| Criscuoli *et al*[17] | 2011 | Full | P[1] | 7 | 21 | Moderate to severe UC | Truelove/ Witts | IHC +, pp65+ | GCV-5 FC-2 | 12 | 2/7 | 4/21 | 2/7 | 4/18 |
| Kim *et al*[12] | 2012 | Full | P[1] | 14 | 17 | Moderate to severe UC | Mayo/ Baron score | HE/IHC/ PCR+ | GCV | hospitalization | 3/14 | 0/17 | NA | NA |
| Roblin *et al*[13][3] | 2011 | Full | P[1] | 8 | 8 | Moderate to severe UC, failure of CS +rescue therapy (IFX/CSA) | Mayo | qPCR, IHC/ HE- | GCV | ⩾ 10 | 1/8 | 2/8 | 3/8 | 2/8 |
| Al-Zafiri *et al*[14][3] | 2012 | Full | R[2] | 7 | 8 | Hospitalized UC | NA | IHC/HE+ | GCV | hospitalization | 3/7 | 0/8 | NA | NA |
| Maruyama *et al*[15] | 2012 | Abstract | R[2] | 4 | 12 | Moderate to severe UC | NA | IHC/HE+ | GCV | Hospitalization | 3/4 | 0/12 | NA | NA |
| Kopylov *et al*[16] | 2013 | Full | R[2] | 7 | 6 | UC | NA | IHC/HE+ | GCV-6 VGCV-1 | 13 | 1/7 | 0/6 | 3/7 | 0/6 |

[1]P: Prospective design; [2]R: Retrospective design; [3]Some of the data was obtained through personal communication; TX: Patients treated with antivirals; C: Patients who did not receive antivirals. PCR: Polymerase chain reaction; qPCR: Quantitative PCR; IHC: Immunohistochemistry; HE: Hematoxylin-eosin staining; pp65: Antigenemia; GCV: Gancyclovir; VGCV: Valgancyclovir; FC: Foscarnet. Short -term outcome- during the initial hospitalization or within 30 d of the diagnosis; long-term outcome- > 3 mo after the initial hospitalization.

**Table 2 Quality of studies on cytomegalovirus in ulcerative colitis assessed according to modified New-Castle Ottawa Scale**

| Total | Outcome | Comparability | Selection | Ref. |
|---|---|---|---|---|
| 1 | | | * | Al-Zafiri *et al*[14], 2012 |
| 3 | * | | ** | Criscuoli *et al*[17], 2011 |
| 4 | * | * | ** | Kim *et al*[12], 2012 |
| 1 | | | * | Kopylov *et al*[16], 2013 |
| 3 | | * | ** | Maconi *et al*[10], 2011 |
| 3 | | * | ** | Maruyama *et al*[15], 2012 |
| 4 | * | * | ** | Omiya *et al*[8], 2010 |
| 4 | * | | *** | Roblin *et al*[13], 2011 |
| 1 | | | * | Zeki *et al*[9], 2010 |
| 24/54 (44.4%) | 4/9 (44.4%) | 4/18 (22.2%) | 16/27 (59.3%) | Total |

In each category, a number of points from 1 (minimal) to 3 (maximal) are attributed by each reviewer. The total score represents a sum of the points achieved at every category.

## DISCUSSION

In this systematic review and MA, we have attempted to compare the outcome of CMV-positive UC patients who were treated with antivirals to that of untreated patients. We did not observe a favorable effect of antiviral therapy for either the primary (short-term colectomy rate) or the secondary (long-term colectomy rate) outcomes. In fact, the patients who had not been treated had a significantly lower risk of a short-term colectomy, and a trend towards improved long-term outcome. However, these results should be interpreted cautiously in view of important confounders and biases that are discussed in detail below.

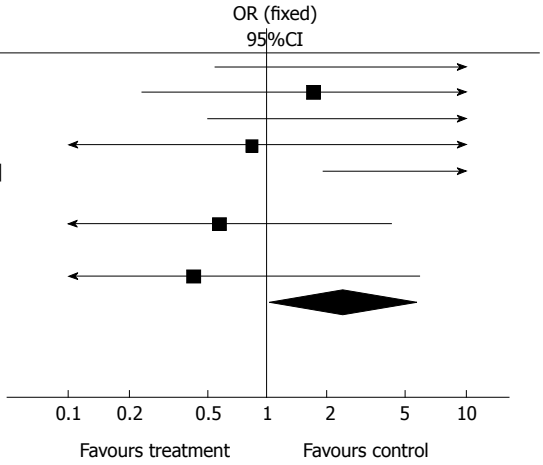
Although CMV infection in IBD patients is frequently described, the vast majority of the studies pertaining to the outcome of this condition are case-reports and case series[18]. Very few prospective studies evaluating the outcomes of such cases have been published, and none employed a randomized blinded design. The true pathological and clinical consequences of the presence of CMV in the colonic tissue in patients with ulcerative

**A**

Colectomy during hospitalization

| Study or sub-category | Antiviral treatment *n/N* | No antiviral *n/N* | Weight % | OR (fixed) 95%CI |
|---|---|---|---|---|
| Al-Zafiri 2012 | 3/7 | 0/8 | 3.64 | 13.22 [0.55, 316.64] |
| Criscuoli 2011 | 2/7 | 4/21 | 19.65 | 1.70 [0.24, 12.17] |
| Kim 2011 | 3/14 | 0/17 | 4.79 | 10.65 [0.50, 226.09] |
| Kopylov 2013 | 1/7 | 1/6 | 12.70 | 0.83 [0.04, 16.99] |
| Maruyama 2012 | 3/4 | 0/12 | 1.15 | 58.33 [1.92, 1770.83] |
| Omiya 2010 | 0/10 | 0/10 | | Not estimable |
| Zeki 2010 | 4/7 | 7/10 | 33.99 | 0.57 [0.08, 4.30] |
| Maconi 2011 | 0/6 | 0/14 | | Not estimable |
| Roblin 2011 | 1/8 | 2/8 | 24.08 | 0.43 [0.03, 5.98] |
| Total (95%CI) | 70 | 106 | 100.00 | 2.40 [1.05, 5.50] |

Total events: 17 (antiviral treatment), 14 (No antiviral)
Test for heterogeneity: $\chi^2$ = 9.55, *df* = 6 (*P* = 0.14), $I^2$ = 37.2%
Test for overall effect: *Z* = 2.07 (*P* = 0.04)




**B**

Colectomy during hospitalization

| Study or sub-category | Antiviral treatment *n/N* | No antiviral *n/N* | Weight % | OR (fixed) 95%CI |
|---|---|---|---|---|
| Criscuoli 2011 | 5/7 | 15/21 | 19.77 | 1.00 [0.15, 6.64] |
| Kopylov 2013 | 3/7 | 0/6 | 2.77 | 10.11 [0.41, 247.48] |
| Omiya 2010 | 3/10 | 0/10 | 3.15 | 9.80 [0.44, 219.25] |
| Roblin 2011 | 5/8 | 2/8 | 6.92 | 5.00 [0.58, 42.80] |
| Suzuki 2010 | 3/15 | 1/3 | 12.30 | 0.50 [0.03, 7.54] |
| Zeki 2010 | 4/7 | 7/10 | 22.80 | 0.57 [0.08, 4.30] |
| Kambham 2004 | 0/3 | 6/8 | 32.29 | 0.50 [0.00, 1.49] |
| Total (95%CI) | 57 | 66 | 100.00 | 1.43 [0.61, 2.96] |

Total events: 23 (Treatment), 31 (Control)
Test for heterogeneity: $\chi^2$ = 9.43, *df* = 6 (*P* = 0.15), $I^2$ = 36.4%
Test for overall effect: *Z* = 0.73 (*P* = 0.47)

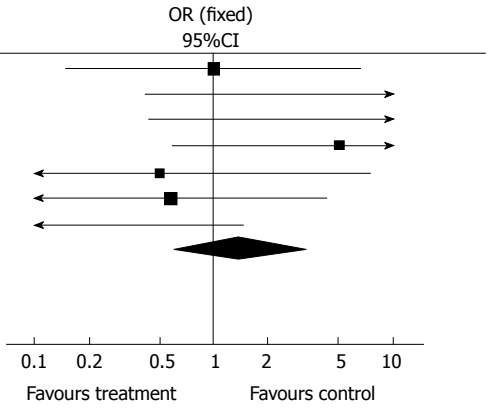



**Figure 2 Colectomy rate in cytomegalovirus infection complicating ulcerative colitis.** A: The rate of colectomy during hospitalization tended to be lower among the patients who did not receive antiviral treatment; B: No significant difference was found in the rate of colectomy during follow-up period.

colitis have been debated for many years, since the initial report by Powell *et al*[19]. Although evidence of CMV infection in the inflamed colonic mucosa of IBD patients is quite common, particularly in steroid-resistant patients[20,21], the actual clinical significance of this finding remains unclear. CMV is trophic for inflamed and replicating tissue, and commonly affects immunosuppressed patients[6,7]. Evidence of viral shedding and replication is often found in IBD patients, almost exclusively in the inflamed mucosa[2]. However, the virus has been shown to disappear from the colonic tissue of UC patients without the administration of antiviral therapy[22]. We will try to address some of the more important controversies on the subject of CMV colitis that are the focus of the present study.

The first issue is the method of diagnosis of CMV infection. Several diagnostic techniques have been described for UC patients. In the past, viral culture was considered a "gold standard" technique for detection of CMV. However, this technique is not sufficiently sensitive and is cumbersome. CMV serology is usually uninformative, as positive CMV IGG is very common. However, a positive CMV IgM is indicative of acute infection[1], and the risk of CMV infection in patients negative for both IgG and IgM is extremely low[23]. None of the studies included in this MA used positive serology as a sole criterion for definition of CMV infection or colitis. Viral particles (pp65 antigen) can be detected in fluid specimens. However, this technique is susceptible to subjective interpretation and can be positive without evidence of colitis[6,24]. Only one of the studies included in this systematic review employed pp65 antigenemia as an indicator of a need for antiviral therapy, along with positive immunohistochemistry[11]. CMV antigenemia testing has generally been replaced by viral DNA detection. CMV DNA can be identified by PCR with a sensitivity of 65%-100% and specificity of 40%-92%[2]. PCR can be positive in patients without colonic involvement, and a correlation with histologic CMV disease has not been universally reported[6,22,25].The presence of CMV in colonic tissue can also be detected by histological methods [hematoxylin-eosin staining (HE), IHC] (Figure 3), as well as PCR. Earlier reports have included steroid-resistant patients with evidence of CMV-induced cytopathic damage on HE staining ("inclusion bodies")[20,26,27]. These patients usually had a severe disease and high

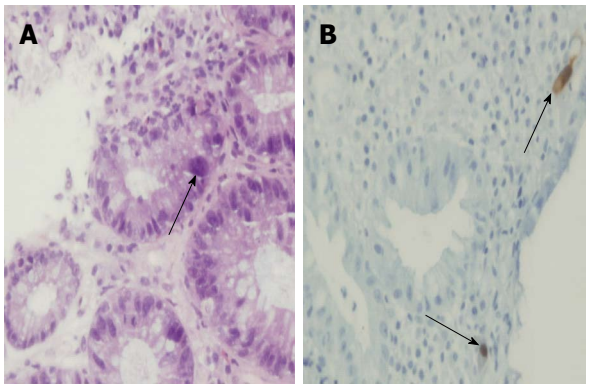



**Figure 3 Cytomegalovirus demonstrated on a colonic biopsy in a patient with ulcerative colitis (arrows).** A: Hematoxylin eosin staining; B: Immunohistochemistry, adapted from [16].

rates of colectomy (up to 67%)[21,26,28]. The detection of inclusion bodies on HE staining is clinically relevant[6] and implies ongoing destruction of the colonic epithelial cells by the virus. Unfortunately, this technique has low sensitivity (10%-87%)[2]. Immunohistochemistry with a monoclonal antibody targeting the early CMV antigen has been reported to improve the diagnostic sensitivity to 78%-93%[2,29]. Current ECCO guidelines recommend the combination of HE staining and IHC for detection of CMV infection in patients with a flare-up of UC[30]. Detection of CMV DNA in the colonic tissue by PCR is highly sensitive, but in the absence of histological evidence of tissue damage possibly represents a remote or latent infection or a low-key viral replication of unclear significance[2]. Recently, reports have been published[13,25] utilizing a quantitative cut-off for a number of CMV particles detected by real-time PCR.

Despite the available evidence, the question as to what test truly defines CMV disease in the colon and thus signifies the need for treatment remains unanswered. There was significant heterogeneity in the definition of CMV among the studies included in this MA. Five[9,10,14-16] defined CMV colitis by presence of a positive IHC staining for CMV. Two studies[8,13] included IHC/HE-negative patients who were positive for CMV in colonic tissue using PCR. Two additional studies[11,12] employed a combination of several techniques to define CMV positivity. A subgroup analysis of studies including only IHC-positive patients did not demonstrate a significant difference in the outcome between the groups (OR = 3.41; 95%CI: 0. 39-29.83, 5 studies).

The standard treatment recommended for CMV colitis employs intravenous gancyclovir (5 mg/kg intravenously every 12 h for 2-3 wk) with a possible switch to oral gancyclovir (1 g/8 h) after clinical improvement for the reminder of the course[2]. These recommendations are not based on experience in IBD, but are derived from data in organ transplant patients[31]. The vast majority of patients in the included studies were initially treated intravenously, usually with gancyclovir, or with foscarnet in gancyclovir-resistant cases. The duration of the intravenous treatment and whether the patients were eventually switched to oral valgancyclovir were not available for majority of the studies. Importantly, administration of both gancyclovir and foscarnet is associated with significant adverse effects (for gancyclovir- bone marrow depression, headaches, somnolence, psychosis, abnormal liver function, fever, and rash; for foscarnet-nephrotoxicity and severe electrolyte abnormalities[2]. These adverse effects were not clearly reported in the majority of the included studies. Two of the studies[9,16] included a total of 3 patients initially treated with oral valgancyclovir. An additional important question that we could not address due to very limited data was whether anti-inflammatory treatment should be stopped when antiviral treatment is instituted.

The included studies were heterogeneous with regards to the severity of disease, as well as the clinical severity score employed (Table 1). Most of the studies included patients with at least moderate colitis, although the scoring system employed was not uniform. In addition, it was not possible to extract data pertaining to individual patient severity categories in order to determine whether severity of underlying disease affected the outcome, as might be expected. The same is true with regards to anti-inflammatory medications used by the patients and the proportion of steroid resistant patients, which could have served as a surrogate marker of severity and the degree of immune suppression. One study[13] included only steroid resistant patients who had failed a rescue medication (infliximab or cyclosporine), while other studies included patients with a wide variety of anti-inflammatory medications. Most of the included studies did not describe a clear strategy supporting the decision to institute or withhold antiviral therapy. Omiya *et al*[8] administered antiviral treatment only to patients with ulcers > 10 mm on colonoscopy. Kim *et al*[12] treated only steroid resistant patients, with steroid-responsive patients having excellent outcome without antiviral treatment. Criscuoli *et al*[17] based the decision to treat on a combination of positive IHC and pp65 antigenemia. Roblin *et al*[13] treated only steroid-resistant patients who were not improved after rescue infliximab or cyclosporine therapy. The rest of the studies treated all CMV-positive patients; however, it appears that patients who quickly responded to anti-inflammatory treatment after hospitalization were less likely to be treated with antivirals and have a favorable outcome without antivirals. On the contrary, patients with a very severe clinical presentation were frequently operated early in the course of the hospitalization, in many cases before their CMV status was established.

There are several important drawbacks to our study. The main weakness was the quality of the included studies. None of the included studies was randomized controlled trial. Only 4 of the studies had a prospective design. The total number of patients included in the analysis is small, reflecting a lack of well-designed large studies. The most significant drawback stems from in-

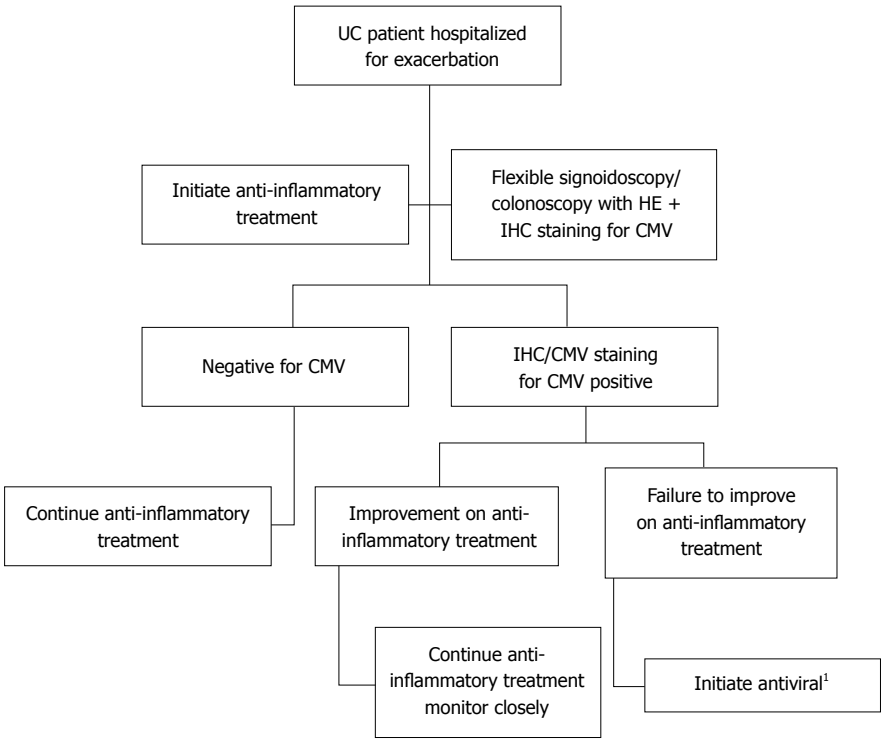



**Figure 4 Proposed algorithm for decision on initiation of antiviral therapy in a ulcerative colitis patient with histological evidence of cytomegalovirus in the colonic mucosa.** [1]Unclear whether antiviral treatment should be tapered/stopped. UC: Ulcerative colitis; HE: Hematoxylin-eosin staining; IHC: Immunohistochemistry; CMV: Cytomegalovirus.

ability to stratify the patients by disease severity, along with an inherent selection bias that resulted in administration of the antiviral therapy to the sicker patients in the majority of the studies. In addition, the definition of CMV infection differed significantly. We have attempted to overcome this heterogeneity in diagnostic criteria by performing a subgroup analysis of studies with a histological definition of CMV colitis. Mortality (2 patients overall) was reported in only 2 of the included studies, precluding any analysis of the impact of antiviral treatment on mortality.

Our MA has several strengths. We employed a stringent inclusion strategy aimed at minimizing selection and publication bias. Primarily, we excluded exclusive colectomy series, as they naturally included only patients who had reached the primary outcome (colectomy). In addition, we excluded the patients in whom the diagnosis of CMV was only available after colectomy, as these patients had never had a chance to receive the treatment. In order to minimize reporting bias, we excluded studies that did not compare patients with and without antiviral therapy, or included 3 or less patients in each arm, as these studies were likely to be biased towards one of the strategies.

In summary, our MA did not demonstrate a benefit of antiviral therapy in CMV-positive patients with UC. The results were not changed if the analysis was restricted to studies using histological (IHC/HE) criteria for diagnosis of CMV. Based on the available literature, we are suggesting an algorithm for management of CMV-positive (as demonstrated by HE/IHC staining) patients hospitalized for UC exacerbation, stratified by clinical response to initial anti-inflammatory treatment (Figure 4). To the best of our knowledge, this is the first attempt to perform a systematic analysis of the multiple studies published on the subject. While the results are hampered by the weakness of the included studies, they do indicate the heterogeneity of this challenging patient cohort, showing that at least some patients with CMV probably do not require antiviral therapy. Thus, these findings underscore the dire need for prospective studies employing stringent clinical, endoscopic and virologic measures to identify the subgroups of patients who are likely to benefit from antiviral therapy, *vs* those who recuperate without this intervention. Such a study should also aim to establish the optimal dose and duration of treatment and the clinical benefit of withholding anti-inflammatory agents.

## COMMENTS

### Background

Cytomegalovirus (CMV) is a very common infection endemic almost ubiquitously. In immunocompromised patients CMV infection can be associated with severe end-organ and systemic infection. CMV presence is frequently detected in the mucosa of patients investigated for exacerbation of ulcerative colitis (UC). However, the impact of CMV infection on the prognosis of UC patients is unclear, and the impact of antiviral therapy on the outcome of these patients has not been well established.

### Research frontiers

Multiple studies describing the outcome of CMV-positive UC patients had been

published in the last twenty years. However, a vast majority of these publications are case series or small case-controlled studies with heterogenous patients cohorts. In this study, we aimed to evaluate the impact of antiviral therapy on the outcome of CMV-positive UC patients using a meta-analysis of the currently available literature.

### Innovations and breakthroughs

The authors did not demonstrate a positive impact of antiviral therapy with gancyclovir on either a short-term (colectomy within 30 d) or long-term (colectomy for the duration of follow-up) outcomes. These results should be addressed with caution due to a low quality of the included studies and important potential biases. The results underline a significant heterogenity of these population, that potentially includes both patie ts with mild disease who do not necessarily require antiviral therapy along with severy ill patients refractory to several lines of anti-inflammatory treatment. This study was underpowered to detect the impact of the disease severity on the outcome of these patient.

### Applications

The results point out that not all of these patients benefit from antiviral therapy, and it is quite possible that patients with good initial response to conventional antiviral treatment may do well on this treatment alone. Well designed randomized controlled studies with stringent disease and outcome definitions are required in order to delineate the optimal treatment strategy for CMV-positive UC patients, and to define the patient subgroups that benefit from such treatment.

### Terminology

Cytomegalovirus - a very common virus that is rarely associated with significant morbidity in healthy individuals, but may be associated with severe complications in immunocompromised patients. It is commonly treated with intravenous gancyclovir

### Peer review

This is an interesting paper on one of the controversial issues in inflammatory bowel disease (IBD) literature on whether to treat or not and under which conditions CMV infection in patients with IBD. The methodology and analysis is technically solid, however a cautious interpretation due to a heavy selection bias is required.

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META-ANALYSIS

# TIPS improves liver transplantation-free survival in cirrhotic patients with refractory ascites: An updated meta-analysis

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## Abstract

**AIM:** To compare the liver transplantation-free (LTF) survival rates between patients who underwent transjugular intrahepatic portosystemic shunts (TIPS) and those who underwent paracentesis by an updated meta-analysis that pools the effects of both number of deaths and time to death.

**METHODS:** MEDLINE, EMBASE, and the Cochrane Library were searched from the inception to October 2012. LTF survival, liver transplantation, liver disease-related death, non-liver disease-related death, recurrent ascites, hepatic encephalopathy (HE) and severe HE, and hepatorenal syndrome were assessed as outcomes. LTF survival was estimated using a HR with a 95%CI. Other outcomes were estimated using OR with 95%CIs. Sensitivity analyses were performed to assess the effects of potential outliers in the studies according to the risk of bias and the study characteristics.

**RESULTS:** Six randomized controlled trials with 390 patients were included. In comparison to paracentesis, TIPS significantly improved LTF survival (HR = 0.61, 95%CI: 0.46-0.82, $P < 0.001$). TIPS also significantly decreased liver disease-related death (OR = 0.62, 95%CI: 0.39-0.98, $P = 0.04$), recurrent ascites (OR = 0.15, 95%CI: 0.09-0.24, $P < 0.001$) and hepatorenal syndrome (OR = 0.32, 95%CI: 0.12-0.86, $P = 0.02$). However, TIPS increased the risk of HE (OR = 2.95, 95%CI: 1.87-4.66, $P = 0.02$) and severe HE (OR = 2.18, 95%CI: 1.27-3.76, $P = 0.005$).

**CONCLUSION:** TIPS significantly improved the LTF survival of cirrhotic patients with refractory ascites and decreased the risk of recurrent ascites and hepatorenal syndrome with the cost of increased risk of HE compared with paracentesis. Further studies are warranted to validate the survival benefit of TIPS in clinical practice settings.



**Key words:** Transjugular intrahepatic portosystemic shunt; Ascites; Paracentesis; Survival; Meta-analysis

**Core tip:** We evaluated the effects of transjugular intrahepatic portosystemic shunts (TIPS) *vs* paracentesis on the liver transplantation-free (LTF) survival in patients with cirrhosis and refractory ascites. Both the number of deaths and the time to death were considered in the present meta-analysis. We found that TIPS significantly improved LTF survival, liver disease-related death, recurrence of ascites, and hepatorenal syndrome; however, TIPS increased the risk of post-TIPS hepatic encephalopathy.

Bai M, Qi XS, Yang ZP, Yang M, Fan DM, Han GH. TIPS im-

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## INTRODUCTION

Refractory ascites is observed in 5%-10% of advanced cirrhosis cases and has a one-year mortality rate of 20%-50%[1-3]. Liver transplantation is the only definitive treatment for these patients, but the procedure is limited by donor liver resources and high cost. Repeated large-volume or total-volume paracentesis with intravenous albumin infusion is currently recommended as the first-line treatment for patients with refractory ascites[4,5]. Although therapeutic paracentesis relieves symptoms rapidly with few technical complications, it does not correct the underlying mechanisms of ascites formation and has negative effects on systemic hemodynamics and renal function[2]. Although surgical portal-caval shunts are effective in the treatment of refractory ascites by reducing the portosystemic pressure gradient (PSG), these shunts have been abandoned because of the high postoperative morbidity and mortality rates[6]. Transjugular intrahepatic portosystemic shunts (TIPS) decompress the PSG and correct the formation of ascites in most cases without the need for general anesthesia, avoiding the risk of major surgery[4,5,7].

Several randomized controlled trials (RCTs) have compared uncovered TIPS with paracentesis in the management of refractory ascites in cirrhotic patients[8-13]. Despite the demonstration by these studies that TIPS was effective in controlling ascites, it was associated with an increased risk of hepatic encephalopathy (HE) and controversial results in survival benefits[8-13]. Based on the data reported in the literature about the five available RCTs[8-12], four previous meta-analyses concluded that TIPS could not significantly decrease patient mortality when compared with paracentesis[6,14-16]. It is notable that all four of these meta-analyses simply combined the number of deaths without considering the effect of the time to death. Thereafter, a meta-analysis by Salerno *et al*[17] pooled individual patient data from four RCTs to overcome this inappropriate survival analysis and demonstrated that TIPS significantly improved liver transplantation-free (LTF) survival. However, the impossibility of collecting individual patient data from all of the identified RCTs is a potential drawback for the meta-analysis conducted by Salerno *et al*[17] and Higgins *et al*[18]. Most likely, the inconsistent conclusions among these meta-analyses were due in part to the hesitation of recommending TIPS as the primary therapy[4,5]. After these meta-analyses, one additional RCT was published in 2011[13]. Thus, it is useful to conduct an updated meta-analysis using an appropriate survival analysis method to evaluate the effect of TIPS on LTF survival in cirrhotic patients with refractory ascites.

The purpose of the present study was to update the previous meta-analyses to evaluate the effect of TIPS on patient survival by appropriate survival analysis. LTF survival was employed as the primary endpoint. Additionally, the causes of death, the number of patients who underwent liver transplantation, the frequency of recurrent ascites, the risk of HE, and the incidence of hepatorenal syndrome were evaluated.

## MATERIALS AND METHODS

### Searching for and selection of studies

Eligible studies were identified by a comprehensive search of MEDLINE, EMBASE, and the Cochrane Library from their inceptions to October 2012. The following key words were used in our searches: ascites, TIPS, paracentesis, and RCT. Reference lists in primary study publications, review articles, editorials, and the proceedings of international congresses were also manually examined.

The following criteria were employed for study selection: (1) study publication: full-text in the English language; (2) study design: RCT; (3) study participants: cirrhotic patients with refractory or recurrent ascites; (4) study interventions: TIPS *vs* large-volume or total-volume paracentesis (with/without intravenous albumin); and (5) one or more of the following outcomes estimated: LTF survival, liver transplantation, cause of death (liver disease-related death or non-liver disease-related death), recurrence of ascites, HE, and hepatorenal syndrome.

### Outcomes and definitions

LTF survival (primary endpoint): patient survival without liver transplantation. Liver transplantation: number of patients who underwent liver transplantation. Liver disease-related death: number of patients who died of liver disease-related causes, including hepatic failure, variceal bleeding, hepatorenal syndrome, and hepatocellular carcinoma. Non-liver disease-related death: number of patients who died of non-liver disease-related causes, such as sepsis, cerebrovascular accident, and cardiac dysfunction[16]. Recurrence of ascites: number of patients who required a new paracentesis after the interventions. HE and severe HE: number of patients who presented with HE after intervention and the number of patients with severe HE (grades Ⅲ/Ⅳ HE according to Conn *et al*[19] or equivalent classification), respectively. Hepatorenal syndrome: number of patients with type 1 or type 2 hepatorenal syndrome.

### Risk of bias assessment

According to the Cochrane risk of bias tool[18], the following six items were used in the assessment of risk of bias: generation of random allocation sequence, concealment of allocation sequence, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, and selective outcome reporting.

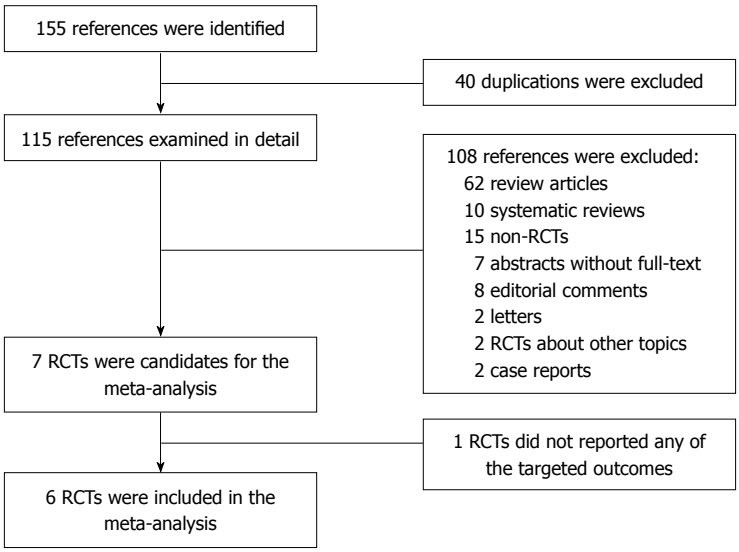


**Figure 1 Randomized controlled trial selection flowchart.** RCT: Randomized controlled trial.

### Data extraction

Determination of trial eligibility and extraction of data were performed independently by two investigators (Bai M and Qi X). Agreements on disagreements were made through discussion. The following data were extracted: patient selection criteria, number of patients screened, number of patients allocated to each study group, detailed information of interventions, study design, duration of follow-up, age, gender, etiology of cirrhosis, Child-Pugh class and score, HE, history of gastrointestinal bleeding, serum bilirubin, serum albumin, serum creatinine, serum sodium, technical results, method of randomization, allocation concealment, blinding, analysis methods, description of drop-outs, and detailed data of outcome measures.

### Statistical analysis

For outcomes reported as time-to-event variables, the HRs are the most appropriate measures to be pooled because both the number of events and the time to events are important[20,21]. The Log (HR) and its standard error for a study are needed to evaluate the pooled HRs. These values were calculated according to the methods described by Parmar *et al*[22] and Tierney *et al*[20]. In summary, randomization ratio, number of analyzed patients, number of observed events, number of expected events, HR and its 95%CI, logrank variance, logrank observed-minus-expected events, and *P* value of logrank test were all used when available. When these variables were insufficient, Kaplan-Meier curves were employed to calculate the Log (HR) and its standard error. These calculations were accomplished by the calculation spreadsheet provided by Tierney *et al*[20]. For outcomes reported as binary variables, the numbers of observed events were extracted and OR were used to evaluate the pooled effect. Heterogeneity was assessed by the $\chi^2$ test and the $I^2$ statistic. Upon confirmation that significant heterogeneity was absent, trials were combined using a fixed-effect model. Otherwise, the results of both fixed-effect and random-effect model were reported. To assess the stability of results, sensitivity analyses were performed on the effects of potential outlier studies according to the risk of bias and the study characteristics. A *P* value of 0.05 was adopted as the criterion for statistical significance. All analyses were performed using Review Manager (RevMan) [Computer program]. Version 5.1. Copenhagen: The Nordic Cochrane Center, The Cochrane Collaboration, 2011.

## RESULTS

Among the 155 identified publications, 40 duplicates were excluded. The remaining 115 papers underwent detailed examination, and 109 were subsequently excluded (Figure 1). Six RCTs including 390 patients reported between 1996 and 2011 were ultimately included in the meta-analysis[8-13].

### Characteristics of the selected trials

The characteristics of the six included RCTs are summarized in Table 1. The control treatment was large-volume paracentesis[9,12,13] and total paracentesis[8,10,11] in three studies each. Intravenous albumin infusion was prescribed after paracentesis in four studies[8,11-13], employed when clinically indicated in one study[9], and used when patients had a creatinine clearance < 60 mL/min in the remaining studies[10]. In five studies[8,9,11-13], refractory ascites was defined according to the criteria reported by the International Ascites Club in 1996[23]. Two trials included patients with recidivant ascites, which was defined as more than three episodes of tense ascites within a 12-mo period despite the administration of standard treatment[9,12].

Five of the studies employed survival as the primary

**Table 1 Characteristics of the included studies**

| Study characteristics | Lebrec *et al*[10] | Rössle *et al*[9] | Ginès *et al*[8] | Sanyal *et al*[11] | Salerno *et al*[12] | Narahara *et al*[13] |
|---|---|---|---|---|---|---|
| Study design | Single-center, RCT | Multi-center, RCT | Multi-center, RCT | Multi-center, RCT | Multi-center, RCT | Single-center, RCT |
| Para | TP with albumin infusion (unclear dose) if creatinine clearance < 60 mL/min | LVP with albumin infusion (8 g/L of ascites removed) when clinically indicated | TP with albumin infusion (8 g/L of ascites removed) | TP with albumin infusion (6-8 g/L of ascites removed) | LVP with albumin infusion (8 g/L of ascites removed) | LVP with albumin infusion (6 g/L of ascites removed) |
| Study population | Cirrhotic patients with refractory ascites | Cirrhotic patients with refractory or recidivant ascites | Cirrhotic patients with refractory ascites | Cirrhotic patients with refractory ascites | Cirrhotic patients with refractory or recidivant ascites | Cirrhotic patients with refractory ascites |
| Definition of refractory ascites | Adequate diuretic and sodium restriction: body weight loss < 200 g/d in 5 d or > 2 tense ascites in 4 mo. | Definition reported in 1996 by International Ascites Club | Definition reported in 1996 by International Ascites Club | Definition reported in 1996 by International Ascites Club | Definition reported in 1996 by International Ascites Club | Definition reported in 1996 by International Ascites Club |
| Exclusion criteria | > 70 yr, HE, severe non-hepatic disease, pulmonary hypertension, PVT/HVT, HCC, active bacterial infection, severe alcoholic hepatitis, biliary obstruction, creatinine > 1.7 mg/dL | HE ⩾ grade 2, bilirubin > 5 mg/dL, creatinine > 3 mg/dL, PVT, hepatic hydrothorax, advanced cancer, failure paracentesis | < 18 or > 75 yr, bilirubin > 10 mg/dL, INR > 2.5, PLT < 40000/mm$^3$, creatinine > 3 mg/dL, HCC, complete PVT, cardiac or respiratory failure, organic renal failure, bacterial infection, and chronic HE | Bilirubin > 5 mg/dL, INR > 2, heart or renal failure, PVT, active bacterial infection, HE > grade 2, severe alcoholic hepatitis, HCC or incurable cancers, GI bleeding within 6 wk | > 72 yr, HE > grade 2, bilirubin > 6 mg/dL, Child-Pugh > 11, creatinine > 3 mg/dL, PVT, HCC, active bacterial infection, cardiac or pulmonary failure, GI bleeding within 15 d | > 70 yr, HE, HCC or other malignancy, PVT, active infection, severe cardiac or pulmonary disease, organic renal disease |
| Primary outcomes | Recurrence of ascites | Transplantation-free survival | Transplantation-free survival | Recurrence of ascites and transplantation-free survival | Transplantation-free survival | Overall survival |
| Secondary outcomes | Overall survival, HE, hemodynamic, liver and renal function | Recurrence of ascites, liver and renal function, HE | Recurrence of ascites, liver and renal function, HE, GI bleeding, HRS | Overall survival, HE, GI bleeding, liver and renal function, quality of life | Recurrence of ascites, HE, GI bleeding, liver and renal function, HRS | Recurrence of ascites, HE |
| Number of patients screened | NR | 155 | 119 | 525 | 137 | 78 |
| Randomized ratio | 1:01 | 1:01 | 1:01 | 1:01 | 1:01 | 1:01 |
| Number of patients randomized (total) | 25 | 60 | 70 | 109 | 66 | 60 |
| Number of participating centers | 1 | 2 | 4 | 6 | 3 | 1 |
| Mean follow-up time (TIPS/Para) | 7.5/12.4 | 45/44 | 9.5/10.8 | 41/38 | 21/15 | 27/13 |

RCT: Randomized controlled trial; NR: Not reported; TIPS: Transjugular intrahepatic portosystemic shunt; Para: Paracentesis; TP: Total paracentesis; LVP: Large-volume paracentesis; HE: Hepatic encephalopathy; PVT: Portal vein thrombosis; HVT: Hepatic vein thrombosis; HCC: Hepatocellular carcinoma; INR: International normalized ratio; PLT: Platelet count; GI: Gastrointestinal; HRS: Hepatorenal syndrome.

endpoint[8,9,11-13], and one study used recurrent ascites as such[10]. The frequencies of recurrence of ascites, HE, and liver transplantation were reported in all studies. Severe HE and hepatorenal syndrome were reported in four[8,10-12] and two trials[8,12], respectively.

### Characteristics of patients in the selected trials

Table 2 summarizes the characteristics of the patients in the six selected trials. The number of randomized patients was at least 60 in all trials except for the study by Lebrec *et al*[10], which only enrolled 25 patients. The percentage of Child-Pugh C patients was 26%-33% in four studies[8-10,13], and 76% in the study by Salerno *et al*[12]. Baseline serum concentrations of bilirubin, albumin, creatinine, and sodium were not significantly different between the TIPS and paracentesis groups in all studies.

### Technical results

Table 3 presents the technical results of the included RCTs. The TIPS technical success rate was at least 89% in five studies[8,9,11-13] but was only 77% in the study by Lebrec *et al*[10]. The average post-TIPS PSGs were 14 mmHg in one study[10], and lower than 12 mmHg in all others[8,9,11-13]. Severe procedure-related complications were reported in three trials, including cardiac arrhythmias[10], hemolytic anemia[8], and cerebrovascular embolism[12]. The proportions of TIPS dysfunction ranged from 30% to 87%. The TIPS-assisted patency rates were higher than 80% in five studies[8,9,11-13]. However, more than 50% of the TIPS patients in the study by Lebrec *et al*[10] did not

**Table 2 Characteristics of the patients in the included studies**

| Patient characteristics | Lebrec *et al*[10] | Rössle *et al*[9] | Ginès *et al*[8] | Sanyal *et al*[11] | Salerno *et al*[12] | Narahara *et al*[13] |
|---|---|---|---|---|---|---|
| Number of randomized patients | 12/13 | 29/31 | 35/35 | 52/57 | 33/33 | 30/30 |
| Age, yr (mean) | 50/52 | 58/61 | 59/56 | 56/52 | 58/60 | 58/61 |
| Percentage of refractory ascites (total) | 100/100 (100) | 58/52 (55) | 100/100 (100) | 100/100 (100) | 72/64 (75) | 100/100 (100) |
| Percentage men (total) | 77/66 (72) | 72/68 (70) | 69/74 (71) | 63/70 (66) | 72/76 (74) | 77/70 (73) |
| Percentage alcohol-induced cirrhosis (total) | 77/83 (80) | 83/74 (78) | 51/60 (56) | 62/58 (60) | 45/39 (42) | 37/33 (35) |
| Percentage Child-Pugh class C (total) | 31/33 (32) | 38/22 (30) | 37/43 (26) | NR | 79/73 (76) | 37/30 (33) |
| Mean Child-Pugh score | 9.3/9.2 | 9.1/8.7 | 9.3/9.2 | 9.2/9.3 | 9.4/9.4 | 8.9/8.9 |
| Percentage with HE (total) | 15/17 (16) | 46/39 (40) | 37/40 (39) | NR | 27/21 (24) | 10/7 (8) |
| Percentage with previous GI bleeding (total) | NR | NR | 34/23 (29) | 23/25 (24) | 18/21 (20) | NR |
| Serum bilirubin, mg/dL | 2.04 ± 0.5/1.57 ± 0.2 | 1.8 ± 1.2/1.8 ± 1.0 | 2.0 ± 0.2/2.4 ± 0.3 | 1.9 ± 1.2/1.9 ± 1.4 | 1.7 ± 0.15/1.9 ± 0.24 | 1.3 ± 0.7/1.4 ± 0.7 |
| Serum albumin, g/dL | 3.0 ± 0.1/3.1 ± 0.2 | 3.5 ± 0.6/3.5 ± 0.4 | 2.8 ± 0.1/3.0 ± 0.1 | 2.9 ± 0.4/2.7 ± 0.4 | 2.9 ± 0.7/2.9 ± 0.8 | 2.7 ± 0.5/2.7 ± 0.6 |
| Serum creatinine, mg/dL | 0.9 ± 0.7/0.9 ± 0.6 | 1.3 ± 0.4/1.4 ± 0.9 | 1.4 ± 0.1/1.4 ± 0.1 | 1.1 ± 0.3/1.0 ± 0.3 | 1.12 ± 0.06/1.15 ± 0.09 | 1.03 ± 0.30/1.03 ± 0.35 |
| Serum sodium, mmol/L | 130 ± 2/130 ± 2 | 130 ± 6/131 ± 6 | 129 ± 1/130 ± 1 | NR | 133 ± 1/133 ± 1 | 134 ± 7/133 ± 5 |

All of the comparisons between groups were not statistically significant ($P > 0.05$) in any of the included studies. TIPS: Transjugular intrahepatic portosystemic shunt; HE: Hepatic encephalopathy; NR: Not reported; GI: Gastrointestinal.

**Table 3 Technical results of the included studies**

| Technical results | Lebrec *et al*[10] | Rössle *et al*[9] | Ginès *et al*[8] | Sanyal *et al*[11] | Salerno *et al*[12] | Narahara *et al*[13] |
|---|---|---|---|---|---|---|
| Successful stent placement (*n*/randomized) | 10/13 (77) | 29/29 (100) | 34/35 (97) | 49/52 (94) | 29/33 (89) | 30/30 (100) |
| PSG change, mmHg | From 20 ± 1 to 14 ± 1 | From 24 ± 6 to 10 ± 4 | From 19.1 ± 0.8 to 8.7 ± 0.4 | From 19.8 ± 4.8 to 8.3 ± 3.6 | From 22.5 ± 1.1 to 8.7 ± 0.6 | From 20.3 ± 4.6 to 8.5 ± 4.7 |
| Severe TIPS procedure-related complications | 1 severe cardiac arrhythmias | None | 3 severe hemolytic anemia | NR | 1 cerebrovascular embolism | None |
| TIPS dysfunction | 3/10 (30) | 13/29 (45) | 13/34 (38) | 34/49 (70) | 12/29 (41) | 26/30 (87) |
| Irreversible stent obstruction | 1/10 (10) | 2/29 (7) | 1/34 (3) | NR | 2/29 (7) | 2/30 (7) |
| TIPS-assisted patency, (*n*/randomized) | 6/13 (46) | 27/29 (93) | 32/35 (91) | > 90% | 27/33 (82) | 26/30 (86) |
| Patients crossed over from paracentesis to TIPS | NR | 10/31 (32) | 3/35 (9) | 2/57 (4) | 11/33 (33) | 6/30 (20) |
| TIPS patency surveillance | Doppler sonography | Doppler sonography | Hepatic vein catheterization if ascites recurred | Angiography | Doppler sonography | Doppler sonography |

Data are expressed as absolute numbers (percentage) or mean ± SD. TIPS: Transjugular intrahepatic portosystemic shunt; PSG: Portosystemic pressure gradient.

have TIPS-assisted patency during the follow-up.

### Risk of bias assessment

All of the studies were unblinded to participants, personnel, and outcome assessment, employed intention-to-treat analysis with description of drop-outs, did not demonstrate the method of generation of random allocation sequence, and reported all of the outcomes described in the methods section (Table 4). The study by Rössle *et al*[9] did not state the concealment of the allocation sequence, while the others concealed the randomization numbers with sealed opaque envelopes (Table 4)[8,10-13].

### LTF survival

LTF survival was directly reported in four studies[8,9,11,12]. Because no patient underwent liver transplantation during the follow-up in the study by Narahara *et al*[13], the LTF survival in this study was certainly equal to the overall survival. Thus, the HRs for LTF survival were available in five RCTs[8,9,11-13]. Compared with the paracentesis group, the LTF survival of the patients in the TIPS group was significantly increased in two studies[12,13], was almost significantly increased in one study[9], and was nearly equivalent in two studies[8,11]. After pooling the five studies with 365 patients, the estimated LTF survival was significantly in favor of the TIPS group using a fixed-effects model (HR = 0.61, 95%CI: 0.46-0.82, $P < 0.001$) without significant heterogeneity ($I^2$ = 19%, $P$ value for heterogeneity = 0.30, Figure 2).

In the study by Lebrec *et al*[10] patient LTF survival was not assessed. Only one patient in the paracentesis group underwent liver transplantation during follow-up.

**Table 4 Risk of bias assessment of the included studies**

| Risk of bias | Lebrec *et al*[10] | Rössle *et al*[9] | Ginés *et al*[8] | Sanyal *et al*[11] | Salerno *et al*[12] | Narahara *et al*[13] |
|---|---|---|---|---|---|---|
| Generation of random allocation sequence (risk) | NR (unclear) | NR (unclear) | NR (unclear) | NR (unclear) | NR (unclear) | NR (unclear) |
| Concealment of allocation sequence (risk) | Sealed opaque envelopes (low) | NR (unclear) | Sealed opaque envelopes (low) | Sealed opaque envelopes (low) | Sealed opaque envelopes (low) | Sealed opaque envelopes (low) |
| Blinding of participants and personnel (risk) | Unblinded (high) | Unblinded (high) | Unblinded (high) | Unblinded (high) | Unblinded (high) | Unblinded (high) |
| Blinding of outcome assessment (risk) | Unblinded (high) | Unblinded (high) | Unblinded (high) | Unblinded (high) | Unblinded (high) | Unblinded (high) |
| Incomplete outcome data (risk) | Intention-to-treat analysis, description of drop-outs (low) | Intention-to-treat analysis, description of drop-outs (low) | Intention-to-treat analysis, description of drop-outs (low) | Intention-to-treat analysis, description of drop-outs (low) | Intention-to-treat analysis, description of drop-outs (low) | Intention-to-treat analysis, description of drop-outs (low) |
| Selective outcome reporting (risk) | All of the outcomes in the methods section were reported in the results section (low) | All of the outcomes in the methods section were reported in the results section (low) | All of the outcomes in the methods section were reported in the results section (low) | All of the outcomes in the methods section were reported in the results section (low) | All of the outcomes in the methods section were reported in the results section (low) | All of the outcomes in the methods section were reported in the results section (low) |

NR: Not reported.

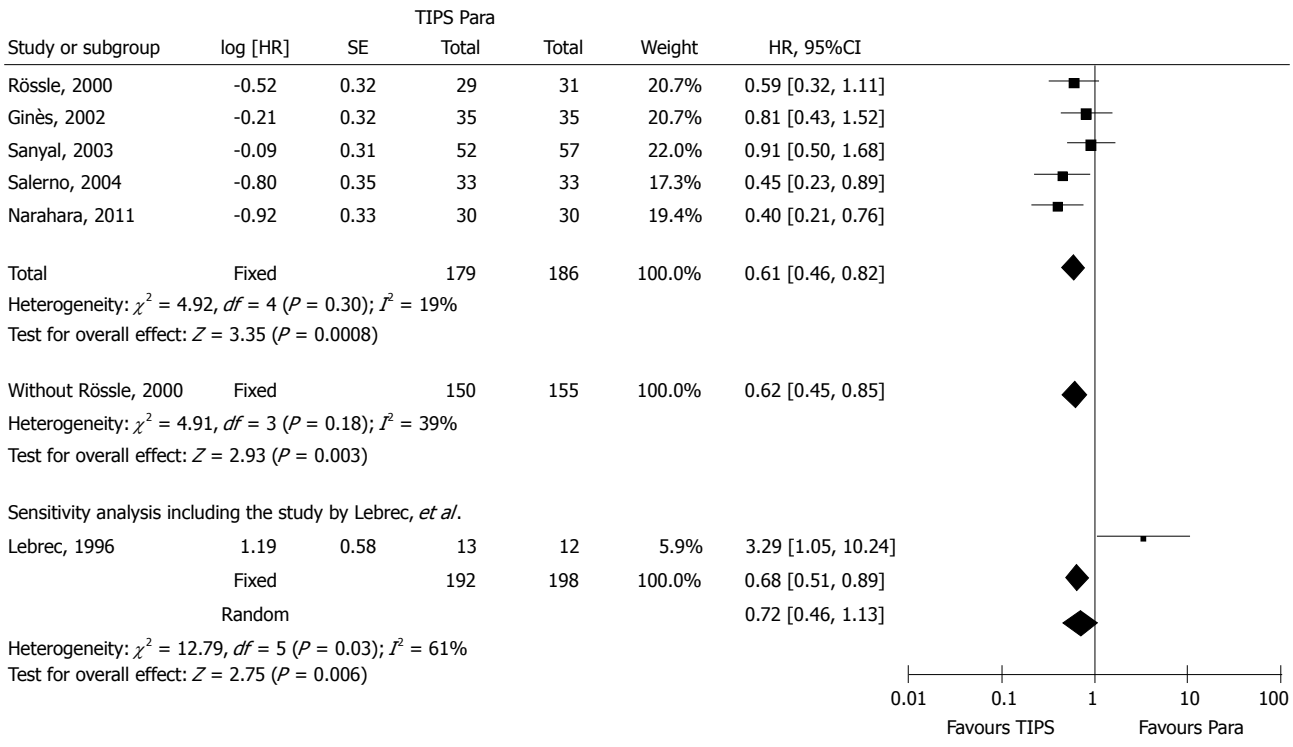


**Figure 2 Liver transplantation-free survival in trials compared transjugular intrahepatic portosystemic shunt with paracentesis.** Forest plots represent HR and 95%CI. TIPS: Transjugular intrahepatic portosystemic shunt.

Thus, the estimated LTF survival of the patients in the paracentesis group would be higher than the overall survival, and the estimated LTF survival of the patients in the TIPS group would be similar to the overall survival. Therefore, we performed an additional sensitivity analysis that included the overall survival of this study to estimate a conservative pooled HR for LTF survival. The results significantly favored the TIPS group that underwent fixed-effect modeling (HR = 0.68, 95%CI: 0.51-0.89, $P$ = 0.006, Figure 2) and tended to favor the TIPS group that underwent random-effect modeling (HR = 0.72, 95%CI: 0.46-1.13, $P$ = 0.16, $I^2$ = 61%, $P$ value for heterogeneity = 0.03, Figure 2).

The subgroup analysis that included the two studies with both refractory and recidivant ascites patients[9,12] demonstrated that LTF survival significantly favored TIPS without significant heterogeneity (HR = 0.52, 95%CI: 0.33-0.83, $P$ = 0.006, $I^2$ = 0%, $P$ value for het-

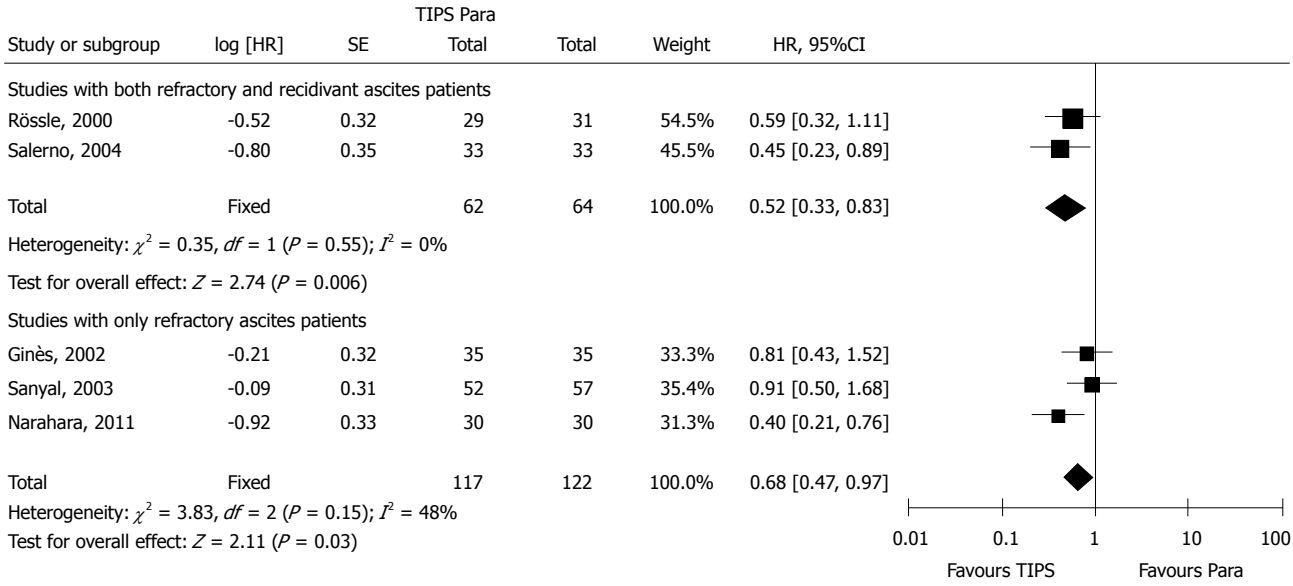


**Figure 3 Subgroup analyses of liver transplantation-free survival in trials compared transjugular intrahepatic portosystemic shunt with paracentesis.** Forest plots represent HR and 95%CI. TIPS: Transjugular intrahepatic portosystemic shunt.

erogeneity = 0.55, Figure 3). Furthermore, the subgroup analysis that included the three studies with only refractory ascites patients[8,11,13] also demonstrated that LTF survival significantly favored TIPS without significant heterogeneity (HR = 0.68, 95%CI: 0.47-0.97, $P$ = 0.04, $I^2$ = 48%, $P$ value for heterogeneity = 0.15, Figure 3).

### Other outcomes

The proportions of liver disease-related death were 30% and 40% in the TIPS and paracentesis groups, respectively. The OR of liver disease-related death was 0.62 without significant heterogeneity (95%CI: 0.39-0.98, $P$ = 0.04, $I^2$ = 31%, $P$ value for heterogeneity = 0.21, Table 5). The pooled proportions of non-liver disease-related death were not significant different between the two groups (OR = 1.27, 95%CI: 0.68-2.38, $P$ = 0.46, $I^2$ = 0%, $P$ value for heterogeneity = 0.64, Table 5).

The proportions of patients who underwent liver transplantation ranged from 0% to 30%[8-12]. No significant difference was observed between the TIPS and the paracentesis groups in the numbers of patients who underwent liver transplantation (OR = 0.94, 95%CI: 0.53-1.67, $P$ = 0.83, $I^2$ = 0%, $P$ value for heterogeneity = 0.94, Table 5).

TIPS was significantly more effective in the reduction of recurrent ascites than paracentesis in four of the included RCTs[8,9,11,12] but was not significantly more effective in the other two studies[10,13]. The overall proportions of patients with recurrent ascites were 51% for the TIPS group and 87% for the paracentesis group (OR = 0.15, 95%CI: 0.09-0.24, $P$ < 0.001). Values for this variable showed no statistically significant heterogeneity ($I^2$ = 2%, $P$ value for heterogeneity = 0.40, Table 5).

HE occurred more frequently in the patients who underwent TIPS procedures (51% *vs* 29%). The OR of any degree of HE between the two groups was 2.95 (95%CI: 1.87-4.66, $P$ < 0.001) without significant heterogeneity ($I^2$ = 11%, $P$ value for heterogeneity = 0.35, Table 5). Patients treated with TIPS presented a significantly higher risk of severe HE than those treated with paracentesis (39% *vs* 23%, OR = 2.18, 95%CI: 1.27-3.76, $P$ = 0.005, Table 5).

Hepatorenal syndrome was assessed in two studies with 136 patients[8,12] and was less frequently observed in the TIPS group (9% *vs* 24%, OR = 0.32, 95%CI: 0.12-0.86, $P$ = 0.02, $I^2$ = 0%, $P$ value for heterogeneity = 0.34, Table 5).

### Potential outlier studies and sensitivity analyses

The study by Lebrec *et al*[10] was considered an outlier for the following two reasons: (1) it was the only trial that employed survival as a secondary endpoint; and (2) it achieved the lowest successful TIPS placement rate, the highest post-TIPS PSG, and the lowest TIPS-assisted patency rate, which indicated a less refined TIPS technique compared with the subsequent trials published 4-15 years later[6,8,9,11-13]. Sensitivity analyses that excluded this trial yielded very similar results (Figure 2, Table 5).

## DISCUSSION

This updated meta-analysis, including appropriate survival analysis of six RCTs, shows that TIPS significantly improves LTF survival and decreases the risk of liver disease-related death in cirrhotic patients with refractory ascites. Additionally, the rates of recurrent ascites and hepatorenal syndrome were significantly reduced, but the risk of HE was significantly increased in the patients who underwent TIPS in comparison to those who underwent paracentesis.

Four previously reported meta-analyses only evaluated the number of deaths without considering the effect

**Table 5 Results of each study and pooled estimations of recurrence of ascites, hepatic encephalopathy, severe hepatic encephalopathy, gastrointestinal bleeding and hepatorenal syndrome by sensitivity analysis**

| Ref. | Liver-transplantation | | | Liver-disease-related mortality | | | Non-liver-disease-related mortality | | | Recurrence ascites | | | HE | | | Severe HE | | | Hepatorenal syndrome | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TIPS | Para | OR (95%CI) | TIPS | Para | OR (95%CI) | TIPS | Para | OR (95%CI) | TIPS | Para | OR (95%CI) | TIPS | Para | OR (95%CI) | TIPS | Para | OR (95%CI) | TIPS | Para | OR (95%CI) |
| Lebrec *et al*[10] | 0/13 | 1/12 | 0.28 (0.01-7.67) | 41438 | 4/12 | 1.71 (0.34-8.68) | 3/13 | 0/12 | 8.33 (0.39-180.36) | 10/13 | 11/12 | 0.30 (0.03-3.41) | 3/13 | 0/12 | 8.33 (0.39-180.36) | 2/13 | 0/12 | 5.43 (0.24-125.59) | - | - | - |
| Rössle *et al*[9] | 0 | 2/31 | 0.52 (0.04-6.04) | 10/29 | 20/31 | 0.29 (0.10-0.84) | 5/29 | 3/31 | 1.94 (0.42-8.99) | 14/29 | 26/31 | 0.18 (0.05-0.60) | 6/29 | 3/31 | 2.43 (0.55-10.82) | - | - | - | - | - | - |
| Ginés *et al*[8] | 7/35 | 7/35 | 1.00 (0.31-3.23) | - | - | - | - | - | - | 17/35 | 29/35 | 0.20 (0.06-0.59) | 27/35 | 23/35 | 1.76 (0.61-5.05) | 21/35 | 12/35 | 2.88 (1.09-7.60) | 3/35 | 11/35 | 0.20 (0.05-0.81) |
| Sanyal *et al*[11] | 16/52 | 17/57 | 1.05 (0.46-2.37) | 13/52 | 13/57 | 1.13 (0.47-2.72) | 5/52 | 6/57 | 0.90 (0.26-3.16) | 22/52 | 48/57 | 0.14 (0.06-0.34) | 22/52 | 13/57 | 2.48 (1.08-5.68) | 15/52 | 10/57 | 1.91 (0.77-4.73) | - | - | - |
| Salerno *et al*[12] | 4/33 | 4/33 | 1.00 (0.23-4.39) | 10/33 | 16/33 | 0.46 (0.17-1.27) | 3/33 | 4/33 | 0.72 (0.15-3.53) | 13/33 | 32/33 | 0.02 (0.00-0.17) | 20/33 | 13/33 | 2.37 (0.88-6.35) | 14/33 | 10/33 | 1.69 (0.61-4.67) | 3/33 | 5/33 | 0.56 (0.12-2.56) |
| Narahara *et al*[13] | 0/30 | 0/30 | - | 8/30 | 13/30 | 0.48 (0.16-1.41) | 9/30 | 8/30 | 1.18 (0.38-3.63) | 22/30 | 27/30 | 0.31 (0.07-1.29) | 20/30 | 5/30 | 10.00 (2.94-34.01) | - | - | - | - | - | - |
| Total | 28/192 | 31/198 | 0.94 (0.53-1.67) | 47/157 | 66/163 | 0.62 (0.39-0.98)[a] | 25/157 | 21/163 | 1.27 (0.68-2.38) | 98/192 | 173/198 | 0.15 (0.09-0.24)[b] | 98/192 | 57/198 | 2.95 (1.87-4.66)[b] | 52/133 | 32/137 | 2.18 (1.27-3.76)[b] | 6/68 | 16/68 | 0.32 (0.12-0.86)[a] |
| Subgroup without the study by Lebrec *et al*, 1996 | 28/179 | 30/186 | 0.98 (0.54-1.77) | 41/144 | 62/151 | 0.56 (0.34-0.91)[a] | 22/144 | 21/151 | 1.11 (0.57-2.14) | 88/179 | 162/186 | 0.14 (0.08-0.24)[b] | 95/179 | 57/186 | 2.86 (1.80-4.54)[b] | 50/120 | 32/125 | 2.10 (1.21-3.67)[b] | - | - | - |

[a]$P < 0.05$, [b]$P < 0.01$; No significant heterogeneity was observed among these meta-analyses ($I^2$ = 0%-31%). All of these meta-analyses were performed under the fixed-effect model. TIPS: Transjugular intrahepatic portosystemic shunt; Para: Paracentesis; HE: Hepatic encephalopathy.

of time to death. All of them showed similar mortality between the TIPS group and the paracentesis group[6,14-16]. According to the PRISMA Statement, the HR is the most appropriate measure to be pooled, because both the number of deaths and the time to the death are important to time-to-event outcomes[21]. For example, in the meta-analysis by D'Amico *et al*[6], the pooled mortality was not significantly different (OR = 0.90, 95%CI: 0.44-1.81). Despite excluding the outlier RCT by Lebrec *et al*[10] which was the only trial that favored paracentesis on survival, the pooled mortality of the remaining four RCTs was still not significantly different (OR = 0.74, 95%CI: 0.40-1.37)[6]. However, if the HRs of the same four RCTs were pooled, the benefit of TIPS on survival was significant (HR = 0.68, 95%CI: 0.50-0.94). This heterogeneity suggested that these two groups of patients had similar numbers of deaths but different survival times. Thus, the HRs were pooled in our present meta-analysis[20,21]. Conversely, because liver transplantation has a very important role in the survival of patients with end-stage liver cirrhosis, this meta-analysis evaluated LTF survival, which isolated the important effect of liver transplantation on survival.

The accumulated LTF survival was available for five of the six included RCTs[8,9,11-13]. All five of these trials evaluated LTF survival using a Kaplan-Meier curve and log-rank test, which gave us facilities to estimate survival difference between the TIPS group and the paracentesis group by pooling the HRs.

After pooling the HRs of the five RCTs, the estimated LTF survival was significantly improved by TIPS compared with paracentesis. Similar improvements were observed when the study by Lebrec *et al*[10] was excluded as an outlier. Furthermore, two of the six RCTs included patients with recidivant ascites (three recurrences of ascites within 12 mo), which represents an earlier stage and has a potentially better prognosis than patients with refractory ascites (recurrence within 4 wk)[23]. Thus, subgroup analyses were performed and showed that TIPS significantly improved LTF survival regardless of if recidivant ascites patients were included or not in the trials.

In a previous study that showed relatively poor survival with TIPS[10], the technical failure rate was more than two-fold higher than the remaining five RCTs (23% *vs* <

11%)[8,9,11-13], and all three of the patients with unsuccessful TIPS procedures died within 3 mo after TIPS. All of these characteristics obviously had a negative contribution to the survival of the TIPS group. We pooled the overall survival of this study in a sensitivity analysis to demonstrate a conservative result, which also showed an improvement of LTF survival in the TIPS group. All of these results suggest that TIPS could improve LTF survival in selected cirrhotic patients with refractory ascites.

An improvement of LTF survival was also reported in a previous meta-analysis that pooled individual patient data from four RCTs[17]. The present study confirmed the effect of TIPS on LTF survival with appropriate survival analysis by pooling data from the literature from six available RCTs. The consistency of survival improvement in these two meta-analyses with different methods makes us more confident that TIPS can do better than paracentesis in the management of refractory ascites.

The improvement of LTF survival in the patients who underwent a TIPS procedure is mostly attributed to the reduction of liver disease-related deaths, especially deaths related to severe complications of portal hypertension. Three studies reported the number of deaths caused by massive variceal bleeding, and all three of the studies showed a lower risk of this type of death in the TIPS group[9-11]. Another cause of the improved LTF survival is that TIPS prolonged the time to liver transplantation, which was reported by two of the enrolled trials[11,12].

TIPS dramatically reduced the incidence of recurrent ascites in the present meta-analysis. This result was consistent with the results of previous meta-analyses[6,14-17]. TIPS procedure also has a positive effect on renal function[2,24]. Thus, it is reasonable that the risk of developing hepatorenal syndrome was reduced by TIPS by more than a half when compared to paracentesis (from 24% to 9%). Because spontaneous bacterial peritonitis and hyponatremia occur more frequently in patients with ascites, TIPS most likely can reduce these events by eliminating the ascites and improving renal function[4,17]. Because hepatorenal syndrome, spontaneous bacterial peritonitis, and hyponatremia are usually associated with high mortality, TIPS most likely improves patient survival by reducing these complications[4,17].

Furthermore, the pooled results showed that TIPS increased the risk of HE and severe HE by almost two-fold in comparison to paracentesis (HE: 51% *vs* 29%, severe HE: 39% *vs* 23%). Similar results were also found in the sensitivity analyses. Although almost all of the post-TIPS HE cases could be successfully managed by medical treatment[8-13,25,26], the reduction level of PSG should be considered with caution, especially in patients with high post-TIPS HE risk (old age, previous HE or high Child-Pugh class)[27-29].

One limitation of this meta-analysis is that all of the included RCTs were designed as open-label trials, which could most likely bias the results by affecting the judgment of actual outcomes, especially subjective outcomes (*i.e.*, HE)[30]. Because blinding is unavailable for these two obviously different interventions, the results presented are most likely the highest quality evidence we can currently obtain. Furthermore, only one of the six RCTs provided raw data by Child-Pugh class[8]. Thus, the subgroup analysis according to liver function is not evaluated in this meta-analysis. However, patient survival was improved by TIPS in both the study including a high proportion of Child-Pugh C patients (76%)[12] and the study including a low proportion of Child-Pugh C patients (33%)[12]. This result indicates that TIPS may be superior to paracentesis regardless of Child-Pugh classes. Additionally, only 48% (median, 21% to 77%, Table 1) of the screened patients could be included in the RCTs, which suggests that studies based on real clinical practice scenarios are needed to validate the universal nature of the results of the present meta-analysis.

In conclusion, this updated meta-analysis of data from six RCTs shows that TIPS significantly improves the LTF survival, the control of refractory ascites, and the prevention of hepatorenal syndrome in patients with cirrhosis and refractory ascites. The increased risk of HE is a major drawback of the TIPS procedure. Further studies based on real clinical practice scenarios are needed.

## COMMENTS

### Background

The survival benefit of transjugular intrahepatic portosystemic shunt (TIPS) in cirrhotic patients with refractory ascites requires further evaluations. Previous meta-analyses of the data reported in the literature considered only the number of deaths, but not the time to death. Furthermore, an additional study on this subject has been recently published. The primary aim of the present study is to compare the liver transplantation-free (LTF) survival between TIPS and paracentesis groups by pooling the effects of both number of deaths and time to death.

### Research frontiers

A meta-analysis was conducted to evaluate the effectiveness of TIPS *vs* paracentesis in patients with cirrhosis and refractory ascites.

### Innovations and breakthroughs

In the present meta-analysis of randomized controlled trials, it was observed that TIPS significantly improved the LTF survival of patients with cirrhosis and refractory ascites. Additionally, TIPS was superior to paracentesis in terms of liver disease-related death, recurrence of ascites, and hepatorenal syndrome. However, patients who underwent TIPS were associated with an increased risk of hepatic encephalopathy (HE) and severe HE.

### Applications

The results of the present meta-analysis suggest that TIPS could potentially be recommended as the first-line treatment for patients with cirrhosis and refractory ascites.

### Terminology

LTF survival (primary endpoint): patient survival without liver transplantation. Liver-disease-related death: number of patients who died of liver-disease-related causes including hepatic failure, variceal bleeding, hepatorenal syndrome, and hepatocellular carcinoma. Recurrence of ascites: number of patients requiring a new paracentesis after the interventions. HE and severe HE: the number of patients presenting with HE after intervention and the number of patients with severe HE (grades Ⅲ/Ⅳ HE or equivalent classification), respectively. Hepatorenal syndrome: number of patients with type 1 or type 2 hepatorenal syndrome.

### Peer review

The effects of TIPS *vs* paracentesis for patients with cirrhosis and refractory ascites have been investigated for more than two decades. The present meta-

analysis included the updated data and found that TIPS could improve the LTF survival rate and alleviate recurrence of ascites, hepatorenal syndrome, and liver disease-related death alone with an increase in HE risk. The paper is exciting and important and brings forth new knowledge.

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CASE REPORT

# Esophageal stent fracture: Case report and review of the literature

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## Abstract

Endoscopic esophageal stent placement is widely used in the treatment of a variety of benign and malignant esophageal conditions. Self expanding metal stents (SEMS) are associated with significantly reduced stent related mortality and morbidity compared to plastic stents for treatment of esophageal conditions; however they have known complications of stent migration, stent occlusion, tumor ingrowth, stricture formation, reflux, bleeding and perforation amongst others. A rare and infrequently reported complication of SEMS is stent fracture and subsequent migration of the broken pieces. There have only been a handful of published case reports describing this problem. In this report we describe a case of a spontaneously fractured nitinol esophageal SEMS, and review the available literature on the unusual occurrence of SEMS fracture placed for benign or malignant obstruction in the esophagus. SEMS fracture could be a potentially dangerous event and should be considered in a patient having recurrent dysphagia despite successful placement of an esophageal SEMS. It usually requires endoscopic therapy and may unfortunately require surgery for retrieval of a distally migrated fragment. Early recognition and prompt management may be able to prevent further problems.



**Key words:** Esophagus; Self-expanding metal stent; Stent complication; Stent fracture; Stent migration

**Core tip:** Esophageal self expanding metal stents are widely used for the treatment of a variety of benign and malignant esophageal conditions. A rare and infrequently reported complication of this procedure is stent fracture and subsequent migration of the broken pieces. There have only been a handful of case reports describing this problem. We report a case of spontaneous fracture of a nitinol esophageal self expanding metal stent, the first reported case from the United States, and review the available literature on this unusual occurrence.

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## INTRODUCTION

Esophageal stent placement is widely used for the palliative treatment of esophageal and gastric cardia cancer. More recently, fully covered esophageal stents have been used for benign esophageal conditions such as refractory stricture, tracheoesophageal fistula, iatrogenic perforation, and post-surgical leaks[1,2]. Esophageal stents have

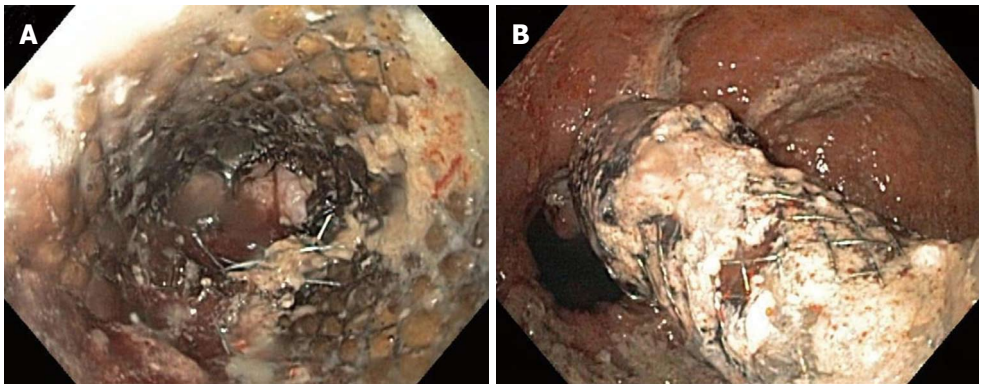


**Figure 1 Endoscopic view of the fractured pieces of the esophageal self expanding metal stent with the proximal fragment embedded in the upper esophagus and the distal migrated fragment wedged in the hiatal hernia.** A: Proximal fragment; B: Distal migrated fragment.

evolved from rigid polyvinyl plastic prostheses ("Celestin tubes") to self-expanding metal stents (SEMS) which may be uncovered, partially covered, or fully covered. SEMS are associated with significantly reduced rates of stent related mortality and morbidity in the form of esophageal perforation and stent migration as compared to plastic stents[3]. SEMS offer a safe and effective means of treatment, which can be placed as an outpatient procedure at low costs[4].

However, SEMS are associated with their own complications. Early complications include chest pain, aspiration from gastroesophageal reflux, bleeding, perforation and stent migration. Delayed complications include stent migration, stent occlusion, tumor ingrowth or overgrowth, stricture formation, reflux, tracheoesophageal fistula formation, bleeding and perforation. Stent migration is the most common complication amongst all, with a frequency of 7%-75%[5,6]. A rare complication of esophageal SEMS is stent fracture described in a handful of case reports, dating as early as 1999 with periodic reports since then. We report a case of a spontaneously fractured nitinol SEMS, which is the first recorded instance in the United States and present a review of all previously published reports of esophageal SEMS fracture.

## CASE REPORT

A 71-year old female with history of squamous cell carcinoma (SCC) of the tongue was treated with radiation and surgical excision 10 years ago. She presented with worsening dyspnea, progressive dysphagia and weight loss. CT scan showed a subcarinal mediastinal mass with extension to the mid-esophagus and metastatic disease to the right upper lung. Endoscopic ultrasound with fine needle aspiration of a lymph node confirmed recurrence of SCC. Chemotherapy was begun and the esophageal lesion was treated with four rounds of liquid nitrogen cryotherapy resulting in shrinkage of the esophageal tumor with symptomatic improvement of her dysphagia.

One year later, she again developed dysphagia; repeat endoscopy showed a malignant stricture from 26 to 31 cm from the incisors. The stricture could only be traversed with an ultra-thin endoscope (outer diameter 5.5 mm) with some resistance. An 18 mm diameter by 12 cm long fully covered Nitinol SEMS (Bonastent, Standard Sci-Tech, Inc, Seoul, South Korea) was successfully deployed across the stricture with its proximal end at 24 cm. Radiographic images of the deployed stent revealed that it was correctly positioned and post-placement fluoroscopy showed complete passage of contrast. The patient also received targeted stereotactic radiotherapy to the right upper lobe of her lung after the stent was placed due to enlarging pulmonary lesions. The radiation field for the lung did not overlap the position of the esophageal stent.

An episode of food impaction within the stent occurred 7 mo after deployment which was cleared endoscopically and she was found to have candida esophagitis with food stasis. During that endoscopy, it was noted that the esophageal stent had Nitinol wire disruption at the 9 o'clock position in the middle of her stent however, the stent lumen was intact. Four months later (11 mo after stent placement), she presented again with dysphagia. Endoscopy showed recurrence of stenosis from the tumor and a complete fracture of the stent at its mid-point with the proximal half embedded into the esophageal lumen and the distal fragment migrated and wedged in a hiatal hernia (Figure 1). Endoscopic dilation was performed using a 10 to 13.5 mm through-the-scope balloon catheter. After dilation, the distal fragment of the broken stent was gently directed down into the stomach. The retrieval lasso at the flared end of the distal fragment was grasped with retrieval forceps and the stent fragment was removed with careful maneuvering. The retrieval lasso at the flared end of the proximal piece was pulled, but the stent could not be moved due to embedding into the esophageal mucosa. The distal end of the proximal fragment was then grasped with an alligator forceps and turned inside out finally allowing successfully removal (Figure 2). A new 18 mm diameter by 10 cm long partially covered Nitinol SEMS (WallFlex, Boston Scientific, Marlborough, MA, United States) was deployed successfully under fluoroscopic and endoscop-

**Figure 2 Endoscopically removed pieces of the fractured esophageal self expanding metal stent.**

ic guidance. The patient had significant improvement of her dysphagia and tolerated an oral diet. The patient died 3 mo later after an episode of massive hemoptysis.

## DISCUSSION

Esophageal SEMS may be made of stainless steel or Nitinol. Nitinol is an alloy made of 55% nickel and 45% titanium, whose name is derived from its composition and its place of discovery: Nickel Titanium Naval Ordnance Laboratory[7]. Nitinol's biocompatibility and the unusual and useful property of shape memory are the reasons for its widespread use in medicine. These stents may be exposed to significant stress-induced fatigue which over a period of time may cause weakening of the metal structure of the stent leading to subsequent fracture and fragmentation.

There have only been 8 published cases of complete esophageal SEMS fracture[8-14] (Table 1). These were all Nitinol stents from different manufacturers and the timing of stent fracture was anywhere from 8 to 40 wk after initial stent placement. The mean patient age was 66 years (range 50-79 years) with 5 male patients, 1 female patient and 2 with gender not reported. The presenting complaint in all cases was dysphagia. They were managed in a variety of different ways. In some cases, the fractured stent pieces were removed endoscopically and a new stent was placed. In other cases, a new stent was placed without removal of the fractured stent. Surgical removal of the distally migrated stent fragment was required in 2 instances. Three of the SEMS were fully covered stents and 5 uncovered. Half of these cases involved the Esophacoil SEMS, which is no longer available. There has been a report of an esophageal fractured stent fragment migrating into the stomach and resulting in the formation of a gastrocolic fistula[13] and thus whenever possible, migrated stents should be retrieved.

Partial Nitinol esophageal stent fracture has been reported more commonly, with 6 publications accounting for 33 patients[9,15-19]. However most of these cases did not need any intervention as it did not affect stent function and only 6 of these cases required placement of new stents through the lumen of the damaged stent due to symptomatic dysphagia, tumor ingrowth and stenosis. Since these stents did not fracture completely, migration of the stent was not an issue.

Fracture of Nitinol SEMS used for the management of malignant obstruction in other parts of the gastrointestinal tract have also been reported. One study reported 2 cases of partial break in the stent wall both in an uncovered and covered enteral Nitinol SEMS placed for symptomatic management of malignant gastric outlet obstruction, with the latter one requiring cutting of the broken part and placement of a second stent to manage food impaction[20]. Two case reports described complete circumferential stent fractures of enteral Nitinol SEMS, one with 2 consecutive fully covered enteral Nitinol SEMS placed for benign duodenal stricture in the same patient[21] and another with an uncovered Nitinol SEMS for duodenal obstruction from periampullary adenocarcinoma[22]. There have been 5 reports describing a total of 14 cases of partial as well as complete fractures of uncovered biliary Nitinol SEMS placed for palliation of malignant biliary obstruction[23-27]. There have also been 3 reports of fracture of colonic Nitinol SEMS placed for malignant large bowel obstruction[28-30].

The possibility of disease or treatment related factors leading to stent fracture have been considered. The use of balloon catheters to dilate Nitinol stents immediately post deployment to guarantee rapid and complete stent expansion to their maximum diameter has been associated with stent fracture in 1 reported case[17]. There have been some instances where esophageal stent breakage has occurred related to laser application to control bleeding from tumor ingrowth. In these cases the authors have postulated that thermal straining of the nitinol alloy could have resulted in stent fracture[18]. In another case series, high dose radiation therapy was associated with fracture of stainless steel tracheobronchial stents[31]. Our patient had also undergone stereotactic radiotherapy to the right upper lobe of her lung after the stent was placed. However the area that was irradiated did not involve the esophagus and hence most likely this did not contribute to the stent fracture.

Nitinol esophageal SEMS are a great improvement over Celestin tubes for the management of malignant dysphagia, mainly due to considerably easier and safer deployment. In addition, they are seeing wider use for management of refractory benign esophageal strictures. However, there are potential complications that may occur following successful deployment. Stent fracture is a rare but potentially dangerous occurrence that should be considered in a patient having recurrent dysphagia after successful placement of an esophageal SEMS. This complication may occur as early as 2 mo after placement. It usually requires endoscopic therapy and may unfortunately require surgery for retrieval of a distally migrated fragment. Early recognition and prompt management may be able to prevent further problems.

**Table 1 Review of all cases of complete esophageal self-expanding metal stent fracture**

| Ref. | Country of Origin | Age (yr)/ Gender | Reason for stent placement | Location | Pre-stent procedures | Initial stent | Stent fracture time after placement | Stent fracture management | Repeat stent placement |
|---|---|---|---|---|---|---|---|---|---|
| Current Case | United States | 71/female | Dysphagia from metastatic esophageal squamous cell carcinoma | Mid esophagus | None | 18 mm × 120 mm, Fully covered Nitinol SEMS - Bonastent, Standard Sci-Tech, Inc, Seoul, South Korea | 45 wk | Proximal piece in the upper esophagus and distal fragment wedged in a hiatal hernia, both removed endoscopically | 18 mm × 100 mm, Partially covered Nitinol SEMS - Wallflex, Boston Scientific, Marlborough, MA, United States |
| Wadsworth *et al*[8], 2010 | United Kingdom | 75/female | Dysphagia from refractory benign esophageal stricture | Distal esophagus | None | 22 mm × 120 mm, Fully covered Nitinol SEMS - EBN stent, Diagmed Healthcare Ltd, Thirsk, United Kingdom | 8 wk | Proximal piece in esophagus removed endoscopically, and distal fragment migrated to the colon, passed rectally | Was under consideration at the time of publication |
| Wiedmann *et al*[9], 2009 | Germany | 69/not reported | Dysphagia from metastastic EAC | Distal esophagus | None | 22 mm × 160 mm, Fully covered Nitinol SEMS - Hanarostent, M.I.Tech Co., Inc, Seoul, South Korea | 20 wk | Proximal piece in esophagus and distal fragment in the gastric antrum, both removed endoscopically | 22 mm × 120 mm, Fully covered Nitinol SEMS - Hanarostent, M.I.Tech Co., Inc, Seol, South Korea |
| Chhetri *et al*[10], 2008 | United Kingdom | 50/male | Dysphagia from EAC | Distal esophagus | Palliative chemotherapy | 18 mm × 110 mm, Fully covered Nitinol SEMS - Choostent, M.I.Tech Co., Inc, Seoul, South Korea | 28 wk | Proximal piece in the esophagus removed endoscopically as it was causing trauma and bleeding, and the distal fragment wedged in the distal two-thirds of the tumor | 18 mm × 120 mm, Partially covered Nitinol SEMS - Ultraflex Boston Scientific, Natick, MA, United States, was placed across the exposed upper end of the tumor and through the prior fractured stent |
| Doğan *et al*[11], 2005 | Turkey | 50/not reported | Dysphagia from metastastic EAC | Distal esophagus | None | 18 mm × 100 mm, Uncovered Nitinol SEMS - Esophacoil, Medtronic InStent Inc., Minneapolis, MN, Unites States | 10 wk | Proximal piece with tumor overgrowth and partial obstruction in the esophagus and distal fragment migrated and left in the stomach | 18 mm, Uncovered Nitinol SEMS - Esophacoil, Medtronic InStent Inc., Minneapolis, MN, United States inserted through the proximal fractured fragment |
| Reddy *et al*[12], 2003 | United Kingdom | 76/male | Dysphagia from EAC at the GE junction | GE junction | Dilation and argon plasma ablation | 18 mm × 100 mm, Uncovered Nitinol SEMS - Esophacoil, Kimal PLC, Uxbridge, United Kindom | 22 wk | Proximal piece in the esophagus with tumor occlusion and the distal fragment in the stomach which subsequently migrated into the right inguinal hernia, removed surgically by enterotomy, followed by herniorrhaphy | Uncovered Nitinol SEMS - Esophacoil, Medtronic InStent Inc., Minneapolis, MN, United States inserted through the proximal fractured fragment |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Reddy *et al*[12], 2003 | United Kingdom | 76/male (same patient) | Dysphagia from EAC at the GE junction | GE junction | None | Size NR, Uncovered Nitinol SEMS - Esophacoil, Kimal PLC, Uxbridge, United Kingdom | 23 wk | Stent fracture in 2 places with proximal piece in the esophagus, middle piece in the stomach and the distal piece in the small intestine, not removed as patient died due to aspiration pneumonia | None |
| Altiparmak *et al*[13], 2000 | Turkey | 52/male | Dysphagia from EAC | NR | None | Size NR, Uncovered Nitinol SEMS - Wallstent, Schneider Inc., Plymouth, MN, United States | 40 wk | Proximal piece in the esophagus and the distal fragment in the stomach causing a gastrocolic fistula with unsuccessful endoscopic removal requiring surgical gastrotomy and fistula repair | None |
| Grimley *et al*[14], 1999 | United Kingdom | 79/male | Dysphagia from refractory anastomotic stricture after resection of EAC | Proximal esophagus | Dilation of anastomotic stricture | 18 mm × 100 mm, Uncovered Nitinol SEMS - Esophacoil, Medtronic InStent Inc., Minneapolis, MN, Unites States | 8 wk | Proximal piece in the esophagus and the distal 2 cm fragment migrated into the stomach, subsequently passed rectally | Partially covered Nitinol SEMS - Ultraflex Boston Scientific, Galway, Ireland, was placed across the exposed upper end of the tumor and through the prior fractured stent |

SEMS: Self-expanding metal stent; EAC: Esophageal adenocarcinoma; GE: Gastroesophageal.

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CASE REPORT

# Pancreaticoduodenectomy following total gastrectomy: A case report and literature review

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## Abstract

We present a case of afferent loop syndrome (ALS) occurring after pancreaticoduodenectomy (PD) in a patient who had previously undergone total gastrectomy (TG), and review the English-language literature concerning reconstruction procedures following PD in patients who had undergone TG. The patient was a 69-year-old man who had undergone TG reconstruction by a Roux-en-Y method at age 58 years. The patient underwent PD for pancreas head adenocarcinoma. A jejunal limb previously made at the prior TG was used for pancreaticojejunostomy and hepaticojejunostomy. Despite normal patency of the hepaticojejunostomy, he suffered from repeated postoperative cholangitis which was brought on by ALS due to shortness of the jejunal limb (15 cm in length). We therefore performed receliotomy in which the hepaticojejunostomy was disconnected and reconstructed using a new Y limb 40-cm in length constructed in a double Roux-en-Y fashion. The refractory cholangitis resolved immediately after the receliotomy and did not recur. Review of the literature revealed the lack of any current consensus for a standard procedure for reconstruction following PD in patients who had previously undergone TG. This issue warrants further attention, particularly given the expected future increase in the number of PDs in patients with a history of gastric cancer.



**Key words:** Pancreaticoduodenectomy following total gastrectomy; Afferent loop syndrome after pancreaticoduodenectomy

**Core tip:** We present a case of afferent loop syndrome occurring after pancreaticoduodenectomy (PD) in a patient who had previously undergone total gastrectomy, and review the English-language literature concerning reconstruction procedures following PD in patients who had undergone total gastrectomy (TG). Review of the literature revealed the lack of any current consensus for a standard procedure for reconstruction following PD in patients who had previously undergone TG. This issue warrants further attention, particularly given the expected future increase in the number of PDs in patients with a history of gastric cancer.

Yokoyama S, Sekioka A, Ueno K, Higashide Y, Okishio Y, Kawaguchi N, Hagihara T, Yamada H, Kamimura R, Kuwahara M, Ichimiya M, Utsunomiya H, Uyama S, Kato H. Pancreaticoduodenectomy following total gastrectomy: A case report and literature review. *World J Gastroenterol* 2014; 20(10): 2721-2724

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## INTRODUCTION

There is no general consensus regarding the method of reconstruction following pancreaticoduodenectomy (PD) in patients who have undergone total gastrectomy (TG). Here, we present a case of afferent loop syndrome (ALS) occurring after PD in a patient who previously underwent TG. The ALS was considered to be due to shortness of the jejunal limb used for hepatico- and pancreatico-jejunostomy and was resolved by revision of the hepaticojejunostomy using a newly-made jejunal limb with sufficient length. In this paper, we detail the post-operative course of this patient, and review the English-language literature concerning methods of reconstruction following PD in patients who have undergone TG.

## CASE REPORT

The patient was a 69-year-old man who had undergone TG for gastric cancer which was reconstructed by the Roux-en-Y method at the age of 58. The patient underwent PD for adenocarcinoma of the pancreas head. The surgery was uneventful. We used the jejunal limb made at the previous TG for pancreaticojejunostomy and hepaticojejunostomy. As a result, the length of the afferent loop was only 15-20 cm (Figure 1).

Although the immediate postoperative course was uneventful, the patient suffered from repeated episodes of cholangitis from 14 d after surgery. This was in spite of normal patency of the hepaticojejunostomy, as confirmed by cholangiography *via* an external biliary stent tube. Based on the findings of an upper gastrointestinal study, which revealed reflux of contrast medium into the biliary tree through the hepaticojejunostomy, ALS was diagnosed. Because the ALS was considered to be due to shortness of the jejunal limb, we performed receliotomy, in which the hepaticojejunostomy was disconnected and reconstructed using a new Y limb 40-cm in length in a double Roux-en-Y fashion 6 wk after the previous surgery (Figure 2). Following receliotomy, no further episodes of cholangitis were observed. The patient was discharged from hospital 4 wk after the receliotomy and remains well without symptoms at 6 mo after receliotomy.

### *Review of the English-language literature*

A PubMed search on July 2013 for articles published since 1965 with the key words "pancreaticoduodenectomy" and "following total gastrectomy" yielded 18 articles in the English-language literature. These publications were all reviewed. Only four papers describing five cases of PD following TG were found, which together with our present case results in a total of six reported cases[1-5] (Table 1). Previous TG was performed for gastric cancer in all six cases. The reconstruction method used following TG was Roux-en-Y in five cases and Billroth Ⅱ in one. The interval between TG and PD ranged from 15 to 132 mo, with a median of 56 mo. PD was performed for pancreatic adenocarcinoma in four cases, pancreatic gastrinoma in one, and distal bile duct carcinoma in one. Of these six, a jejunal limb already constructed in the previous TG was used for pancreatic and biliary reconstruction in three cases (including our own case). In the other three cases, a newly-made jejunal limb was used for pancreatic and biliary reconstruction in a Roux-en-Y fashion. ALS was observed in two of the three cases in which a jejunal limb made in a previous TG was used for pancreatic and biliary reconstruction.

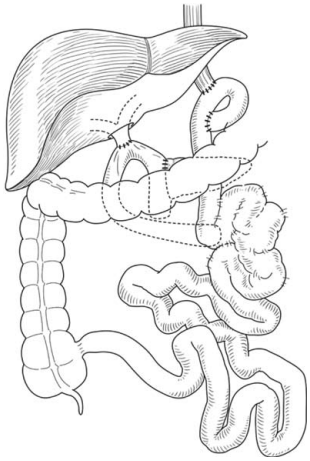

**Figure 1 Hepaticojejunostomy was performed using the same previous afferent loop after total gastrectomy.** The afferent loop was only 15-20 cm long.

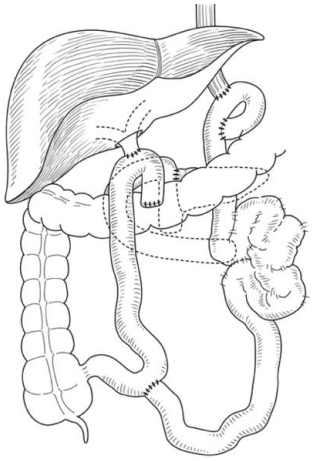

**Figure 2 Receliotomy was performed where the hepaticojejunostomy was disconnected and reconstructed using a new Y limb 40 cm in length and a double Roux-en-Y technique.**

## DISCUSSION

Pancreaticoduodenectomy in patients with a history of major abdominal surgery is a challenging task. Notably, PD in patients with previous TG is difficult, and can be

**Table 1 List of patients who underwent pancreaticoduodenectomy after total gastrectomy**

| No. | Gastrectomy | | | | Pancreaticoduodenectomy | | | | | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Age | Indication | Type of Gastrectomy | Anastomosis | Age | Interval (mo) | Indication | Reconstruction | Operative time (min) | Complication | |
| 1 | 65 | Gastric cancer | Total | Roux-en-Y | 69 | 43 | Pancreatic cancer | Roux-en-Y[2] | 509 | Afferent loop syndrome | [1] |
| 2 | 45 | Gastric ulcer | Total | Roux-en-Y | 46 | 15 | Pancreatic Gastrinoma | New-Roux-en-Y | 503 | None | [2] |
| 3 | 61 | Gastric cancer | Total[1] | Roux-en-Y | 71 | 120 | Pancreatic cancer | New-Roux-en-Y | N/A | Pancreatic fistula | [3] |
| 4 | 64 | Gastric cancer | Total | Roux-en-Y | 68 | 48 | Bile duct cancer | Roux-en-Y[2] | 445 | None | [4] |
| 5 | 58 | Gastric cancer | Total | Roux-en-Y | 69 | 124 | Pancreatic cancer | Roux-en-Y[2] | 568 | Afferent loop syndrome | Our case |
| 6 | 36 | Gastric cancer | Total | Billroth-Ⅱ | 56 | 56 | Pancreatic cancer | New-Roux-en-Y | 672 | None | [5] |

[1]Total gastrectomy, distal pancreatectomy, and splenectomy; [2]Previous afferent loop. N/A: Not available.

limited by adhesions and anatomical complexity around the pancreas subsequent to the previous TG procedure. In our patient, we performed PD with resection of the duodenal part of a previous afferent loop, and used the remaining part of the afferent loop for pancreatico- and hepaticojejunostomy. We applied this procedure to reduce the number of intestinal anastomoses. As a result, however, the jejunal limb used was markedly short, resulting in the development of refractory cholangitis due to ALS.

There is no consensus regarding the standard procedure for reconstruction following PD for patients with previous TG. Furthermore, our patient was seriously affected by a resultant mistake in choosing a reconstruction procedure following PD. We therefore conducted a review of the literature, with a focus on reconstruction procedures following PD in patents who had previously undergone TG. This review demonstrated that it is difficult to state that usage of a jejunal limb previously made at a prior TG should be avoided, as only a few cases of PD in patients with previous TG have been reported, although ALS, which is reportedly rare after PD[2], was observed in two of three cases where an already-made jejunal limb was used for pancreatic and biliary reconstruction. In contrast, ALS was not observed in any of the other three cases in which a jejunal limb for reconstruction was newly made. Furthermore, ALS in our patient was remedied by receliotomy, in which the hepaticojejunostomy was disconnected and reconstructed using a new Y limb 40-cm in length, which was constructed in a double Roux-en-Y fashion. We therefore consider that jejunal limbs should be reconstructed following PD in patients with previous TG in which the jejunal limb is markedly short.

Because the outcomes of treatment for gastric cancer, for which gastrectomy is most commonly performed, have improved, the number of PD procedures in patients with previous gastrectomy is expected to increase[6,7]. We hope that high-volume centres will conduct studies of sufficient size to allow the establishment of a standard reconstruction procedure for this condition.

## COMMENTS

### Case characteristics

Although the early postoperative course was uneventful, the patient suffered from repeated episodes of cholangitis from 14 d after surgery.

### Clinical diagnosis

A case of afferent loop syndrome (ALS) occurring after pancreaticoduodenectomy (PD) in a patient who had previously undergone total gastrectomy (TG).

### Imaging diagnosis

Based on the findings of an upper gastrointestinal study, which revealed reflux of contrast medium into the biliary tree through the hepaticojejunostomy, ALS was diagnosed.

### Treatment

Because ALS was considered to be due to shortness of the jejunal limb, we performed receliotomy, in which the hepaticojejunostomy was disconnected and reconstructed using a new Y limb 40 cm in length in a double Roux-en-Y fashion 6 wk after the previous surgery.

### Related reports

Only four papers describing five cases of pancreaticoduodenectomy following total gastrectomy were found, which together with the present case results in a total of six reported cases.

### Experiences and lessons

This study demonstrates that jejunal limbs should be reconstructed following PD in patients with previous TG in which the jejunal limb is markedly short.

### Peer review

The high-volume centres will conduct studies of sufficient size to allow the establishment of a standard reconstruction procedure for PD following TG.

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CASE REPORT

# Huge undifferentiated carcinoma of the pancreas with osteoclast-like giant cells

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## Abstract

Undifferentiated carcinoma of the pancreas with osteoclast-like giant cells (OGCs) is very rare, less than 1% of all pancreatic malignancies, and shows worse prognosis than that of invasive ductal adenocarcinoma of the pancreas. We present a case of en bloc resection for a huge undifferentiated carcinoma with OGCs that invaded the stomach and transverse mesocolon. A 67-year female was admitted for left upper quadrant pain and computed tomography demonstrated a mass occupying the lesser sac and abutting the stomach and pancreas. There were no distant metastases and the patient underwent subtotal pancreatectomy with splenectomy, total gastrectomy, and segmental resection of the transverse colon. Histopathological examination confirmed an 11 cm-sized undifferentiated carcinoma of the pancreas with OGCs. Immunohistochemical staining revealed reactivity with pan-cytokeratin in adenocarcinoma component, with vimentin in neoplastic multinucleated cells, with CD45/CD68 in OGCs, and with p53 in tumor cells, respectively. The patient had suffered from multiple bone metastases and survived 9 mo after surgery. This case supports the ductal epithelial origin of undifferentiated carcinoma with OGCs and early diagnosis could result in favorable surgical outcomes. Investigations on the surgical role and prognostic factors need to be warranted in this tumor.



**Key words:** Carcinoma; Giant cell; Pancreas; Prognosis; Treatment outcome

**Core tip:** Undifferentiated carcinoma of the pancreas with osteoclast-like giant cells (OGCs) is very rare and shows a poor prognosis. A 67-year female underwent subtotal pancreatectomy, total gastrectomy, and segmental resection of the transverse colon for a mass occupying the lesser sac and abutting the stomach and pancreas. Histopathological examination confirmed an 11cm-sized undifferentiated carcinoma of the pancreas with OGCs. The patient had suffered from multiple bone metastases and survived 9 mo after surgery. This case supports the ductal epithelial origin of undifferentiated carcinoma with OGCs and early diagnosis could result in favorable surgical outcomes.

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## INTRODUCTION

Undifferentiated carcinoma of the pancreas is a rare and aggressive tumor and a variety of other terms, such as osteoclast-like, osteoclastic or pleomorphic giant cell tumor, anaplastic carcinoma, pleomorphic (giant cell) carcinoma, sarcomatoid carcinoma, and spindle cell carcinoma, have been used to describe this type of tumor. These various terms are put together into a single category designated as undifferentiated carcinoma of

the pancreas in the current WHO Classification of the tumours despite their histological differences[1]. In addition, as undifferentiated carcinoma of the pancreas may accompany osteoclast-like giant cells (OGCs) that are suggested to be a reactive non-neoplastic histiocytic origin, this WHO Classification separates undifferentiated carcinoma with OGCs from plain undifferentiated carcinoma, implying there are a few clinical and histopathological distinctions between them.

Undifferentiated carcinoma of the pancreas has been reported a rare tumor[2,3] and OGCs-accompanying undifferentiated carcinoma of the pancreas an extremely rare tumor, less than 1% of all pancreatic malignancies[4]. This very rare undifferentiated carcinoma of the pancreas with OGCs shows worse prognosis than that of invasive ductal adenocarcinoma of the pancreas[5-8], because it is frequently found to be unresectable at diagnosis due to advanced stages[9,10] and tends to early recur even after complete surgical resection[4,11,12]. Correspondingly, median or average survival of patients with undifferentiated carcinoma of the pancreas with OGCs has been reported less than 1 year with few exceptions[4,7,13-16].

There have been relatively few reports, primarily based on case reports, regarding the clinical and histopathological features of this fatal tumor in literatures. In this report, we present a case of a huge undifferentiated carcinoma of the pancreas with OGCs which directly invaded the stomach and transverse mesocolon but was successfully en bloc resected, and review the literature with emphasis on the histogenesis and surgical outcomes.

## CASE REPORT

A 67-year female was admitted to the department of gastroenterology for left upper quadrant pain of two months. The pain was aggravated by diet but no nausea or vomiting was reported. The patient presented weigh loss of about 3 kg over two months. She had no noticeable past medical history and underwent total vaginal hysterectomy and appendectomy 10 years ago. No allergies or significant social or family history was noted. She had taken medicine for esophagitis for two months. On physical examination, chronically ill-looking appearance was observed and vaguely palpable abdominal mass with mild deep tenderness was detected.

Abnormal laboratory results were decreased hemoglobin at 11.3 g/dL (reference range, 12.0 to 16.0 g/dL), elevated amylase at 157 U/L (reference range, 28 to 100 U/L), and elevated CA 19-9 at 73.2 U/mL (reference range, 0 to 37 U/mL). Lipase, bilirubin and transaminases were normal.

Gastric endoscopy demonstrated a huge extrinsically compressing mass mainly against the lesser curvature side of the antrum and body. CT scans showed an about 10cm-sized huge mass occupying the lesser sac, involving parenchyma of the pancreatic body and neck portion, and abutting the gastric posterior wall and duodenal second portion (Figure 1A). This mass looked like arising from the pancreas and compressing the main hepatic artery and portal vein. Endoscopic ultrasonography (EUS) revealed a heterogeneous and poorly demarcated mass with small central cystic lesions between the pancreas body and stomach; the mass seemed to be originated from the pancreas body and abutted on the gastric wall; portal vein and common bile duct were intact and pancreatic duct dilatation was not definite. EUS-guided fine needle aspiration demonstrated malignant tumor, suggestive of undifferentiated carcinoma of the pancreas. As no definite distant metastases were found on PET-CT, the patient was transferred for surgery.

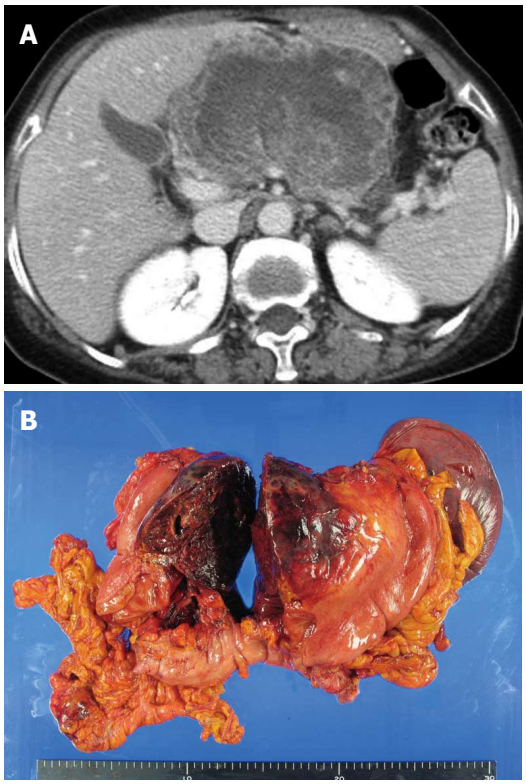


**Figure 1 Huge undifferentiated carcinoma with osteoclast-like giant cells.** A: Computed tomography scans revealed the tumor occupying the lesser sac; B: Tumor arose from the pancreas body and invaded directly the stomach wall and transverse mesocolon.

On intraabdominal exploration, no metastatic peritoneal nodules were detected. The mass arose from the pancreas and directly invaded the lesser curvature of stomach and the mid portion of transverse mesocolon. Fine dissection was initiated between the mass and major hepatic inflow vessels in order to investigate curative resectability. After confirming complete dissection between them, further dissection proceeded and finally en bloc resection was performed through subtotal pancreatectomy with splenectomy, total gastrectomy, and segmental resection of the transverse colon (Figure 1B).

Histopathological examination confirmed an 11cm-sized undifferentiated carcinoma of the pancreas with OGCs, which extended beyond the pancreas to the stomach wall and transverse mesocolon. The tumor was predominantly composed of spindle-shaped, highly

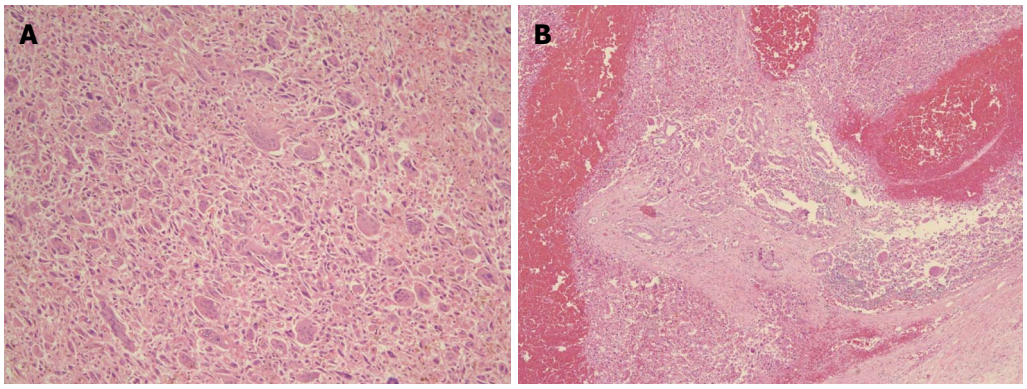


**Figure 2 Histopathological characteristics.** A: Tumor was composed of highly pleomorphic neoplastic cells and non-neoplastic osteoclast-like giant cells. Hematoxylin and eosin (H and E), × 100; B: Ductal adenocarcinoma component was also found in some areas. H and E, × 40.

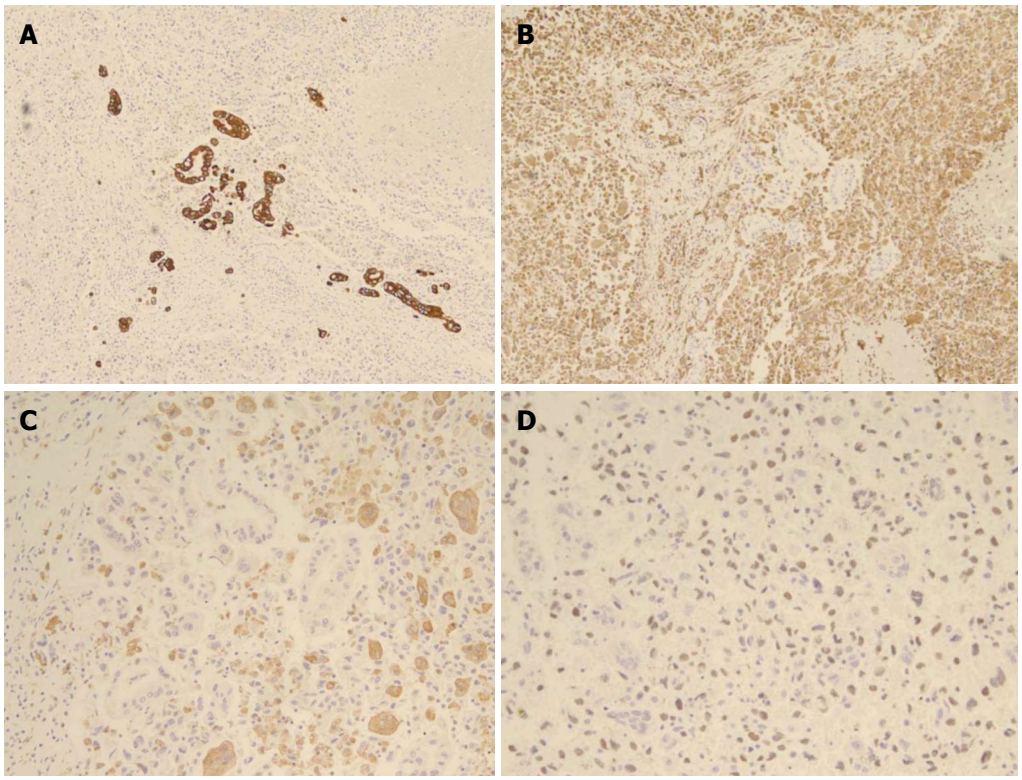


**Figure 3 Immunohistochemical staining.** A: Reactivity with cytokeratin in ductal adenocarcinoma component; B: Reactivity with vimentin in pleomorphic neoplastic cells; C: Reactivity with CD68 in osteoclast-like giant cells; D: Reactivity with p53 in tumor cells.

pleomorphic, neoplastic, mono- or multinucleated cells, as well as non-neoplastic multinucleated OGCs, sporadically intermingled with neoplastic cells (Figure 2A). Characteristically, ductal adenocarcinoma component was found in some areas, providing evidence of ductal cell origin of this undifferentiated carcinoma with OGCs (Figure 2B). Additional reports of extensive hemorrhage and necrosis of the tumor (up to 80%), negative resection margins, no lymphovascular invasion, and nodal metastases (2/30) were given. Immunohistochemical staining revealed reactivity with pan-cytokeratin in adenocarcinoma component, with vimentin in undifferentiated carcinoma component (neoplastic multinucleated cells), with CD45/CD68 in OGCs, and with p53 in tumor cells, respectively. Non-neoplastic OGCs did not show reactivity with vimentin or p53 (Figure 3).

The patient commenced soft diet on postoperative day (POD) 6$^{th}$ and was discharged on POD 19$^{th}$ without significant complications. She had been followed free of recurrence for a half year. Seven months after surgery the patient complained lower back pain and lumbar MRI revealed multiple mass lesions of myeloinfiltrative pattern in the thoracolumbar spines and pelvic bones. She had suffered from multiple bone metastases and showed 9-mo survival after surgery.

## DISCUSSION

Undifferentiated carcinoma of the pancreas with OGCs is characterized by a dual component of undifferentiated

**Table 1 Surgical cases diagnosed with undifferentiated carcinoma of the pancreas with osteoclast-like giant cells**

| Ref. | Year | Case | Age (yr) | Sex | Location | Size (cm) | Operations | Outcomes |
|---|---|---|---|---|---|---|---|---|
| Molberg *et al*[4] | 1998 | 7 | 43-88 | 5F, 2M | Head, tail | 5-14 | PD, DP | DOC 1 mo-NED 14 yr |
| Carvounis *et al*[24] | 2003 | 1 | 70 | F | Head and neck | 7 | PD | AWD 9 mo |
| Bedioui *et al*[14] | 2004 | 1 | 72 | F | Head | 6 | PD | NED 18 mo |
| Charfi *et al*[25] | 2006 | 1 | 65 | F | Tail | 10 | DP | DOD 12 mo |
| Tezuka *et al*[16] | 2006 | 1 | 68 | F | Head | 0.7 | PD | NED 22 mo |
| Jang *et al*[10] | 2006 | 1 | 75 | M | Body and tail | 18 | Palliative DP | NA |
| Hirano *et al*[12] | 2008 | 1 | 26 | F | Body and tail | 11 | DP | NED 8 mo |
| Manduch *et al*[26] | 2009 | 1 | 66 | M | Head | 9.5 | PD | DOD 12 mo |
| Daum *et al*[27] | 2010 | 1 | 71 | F | Body and tail | 17 | DP | NA |
| Mannan *et al*[22] | 2010 | 1 | 40 | F | Head and neck | 4 | PD | NA |
| Maksymov *et al*[23] | 2011 | 1 | 68 | F | Uncinate process | 2 | PD | NED 14 mo |
| Wada *et al*[11] | 2011 | 1 | 59 | M | Tail | 14 | DP and total gastrectomy | DOD 4 mo |
| Hur *et al*[28] | 2011 | 1 | 77 | F | Tail | 10 | DP and left hemicolectomy | DOC 3 mo |
| Yoshioka *et al*[13] | 2012 | 1 | 74 | F | Body | NA | DP | DOD 19 mo |

M: Male; F: Female; PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy; DOC: Died of other causes; NED: No evidence of disease; AWD: Alive with disease; DOD: Died of disease; NA: Not available.

carcinoma cells (neoplastic mono- or multi-nucleated cells) and multinucleated OGCs, mimicking giant cell tumor of bone. Although there have been few reports suggesting origins of undifferentiated carcinoma of the pancreas from acinar cells, mesenchymal cells, undifferentiated precursor or stem cells, mucinous cystic neoplasms, and ductal cells[9], a duct epithelial origin is now established and this tumor has been recognized as a variant of ductal adenocarcinoma of the pancreas in the most recent WHO classification[1]. Verbeke *et al*[17] confirmed the duct epithelial origin of undifferentiated carcinoma of the pancreas in their letter to the editor by demonstration of (1) foci of conventional ductal adenocarcinoma component; (2) occasional association with mucinous cystic neoplasia; (3) cytokeratin expression in at least some of the pleomorphic tumour cells; and (4) K-ras mutations in the pleomorphic tumour cells, with identical mutations identified in associated foci of conventional ductal adenocarcinoma or intraductal neoplastic lesions. In this case, histopathological analysis of the resected specimen revealed the coexistence of adenocarcinoma component and undifferentiated carcinoma component with reactivity with vimentin, suggesting that the tumor originated from pancreatic ductal cells with mesenchymal differentiation. These data provide evidence to support the epithelial origin of these neoplastic components.

Non-neoplastic OGCs in undifferentiated carcinoma of the pancreas is the histopathological hallmark. OGCs present in this tumor are consistently found to be of a reactive mesenchymal nature, characterized by the lack of morphological atypia, proliferative activity, and K-ras and p53 abnormalities[17]. In contrast to the established origin of undifferentiated carcinoma of the pancreas, there have been controversies regarding the origin of OGCs. Proposed origins of OGCs have included epithelial, histiocytic, or mesenchymal metaplasia[18]. However, their nuclear features, lack of reactivity with epithelial markers, and CD68 and lysozyme reactivity are indicative of a histiocytic origin[4]. These characteristic giant cells were suggested to result from fusion of mononuclear histiocytes/marcophages attracted by growth or chemotactic factors produced by the neoplastic cells[19]. The presence of OGCs in this tumor may reflect clinical significance, for example, a better prognosis or response to adjuvant therapy compared to undifferentiated carcinoma without OGCs. In this context, the most recent WHO Classification might have separated undifferentiated carcinoma with OGCs from undifferentiated carcinoma without OGCs, though comments on clinical differences between both tumors lack[20]. Some authors reported that undifferentiated carcinoma with OGCs might have a more favorable prognosis than pancreatic ductal adenocarcinoma[21] or undifferentiated carcinoma without OGCs[22,23].

A few cases diagnosed with undifferentiated carcinoma of the pancreas with OGCs have been reported and are summarized in Table 1. Surgical outcomes in patients with this unusual tumor have been disappointing. They are even worse than those of fatal invasive ductal adenocarcinoma of the pancreas[5-7]. Although few long-term survivors have been reported, even more than 10-year survivors[4,7,29], most of the patients in case reports showed early recurrence and rapid progression even after complete surgical resection and died of tumor within 1 year[4,11,12]. The patient in this case also suffered from multiple bone metastases without definite evidence of local recurrence and survived only 9 mo after curative resection. Concerning the surgical role, there have been no reports in undifferentiated carcinoma of the pancreas with OGCs. Instead, some authors recommended appropriate surgery for pleomorphic carcinoma of the pancreas with favorable characteristics, absence of invasion of adjacent organs and distant metastases[30], but others did not due to poor surgical outcomes[31]. Interestingly, *in situ* as well as early-stage undifferentiated carcinomas of the pancreas with OGCs have been reported[9,16,23]. The tumor size ranged from 2.0 to 5.3 cm and postoperative outcomes, described in only one case[16], were satisfactory with no evidence of tumor recurrence or metastasis. Accordingly, early diagnosis and subsequent complete

resection, although it is exceedingly uncommon, could be only chance to cure this fatal tumor. Tumor size tends to be small in an early stage and smaller-sized undifferentiated carcinomas with OGCs have showed favorable surgical outcomes in some cases[14,16]. However, overall tumor size has not been reported to be a reliable prognostic indicator[7], because patients with even a large undifferentiated carcinoma could show long-term survival[4]. Investigations on the surgical role and prognostic factors need to be warranted based on multi-center cooperation and large cohort of patients with undifferentiated carcinoma with OGCs.

In summary, undifferentiated carcinoma of the pancreas with OGCs originates from duct epithelial cells and is now recognized as a variant of ductal adenocarcinoma of the pancreas. The present case supports evidence of tumor origin from pancreatic ductal cells. Non-neoplastic OGCs in undifferentiated carcinoma derive from histiocytic lineage and may reflect better clinical courses compared to tumor without OGCs. Surgical outcomes in patients with undifferentiated carcinoma with OGCs have been disappointing. Early diagnosis and subsequent complete resection could be only chance to cure this fatal tumor but tumor size may not prognostic indicator.

## ACKNOWLEDGMENTS

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## COMMENTS

### Case characteristics

A 67-year female complained left upper quadrant pain, lasting 2 mo and aggravated by diet.

### Clinical diagnosis

The patient presented 3 kg-weight loss over two months and vaguely palpable abdominal mass with mild deep tenderness was detected.

### Differential diagnosis

Malignant tumor was suspected and abdominal computed tomography (CT) scan was firstly performed.

### Laboratory diagnosis

CBC, liver function profile, pancreatic enzymes, and tumor markers were tested and resultantly amylase and CA 19-9 were slightly (about two fold) elevated.

### Imaging diagnosis

CT scan showed an about 10 cm-sized huge mass arising from the pancreas, compressing the adjacent major vessels, and abutting the gastric posterior wall and duodenal second portion.

### Pathologic diagnosis

Endoscopic ultrasonography-guided fine needle aspiration biopsy demonstrated malignant tumor, suggestive of undifferentiated carcinoma of the pancreas.

### Treatment

The patient underwent en bloc resection through subtotal pancreatectomy with splenectomy, total gastrectomy, and segmental resection of the transverse colon, but later she refused adjuvant therapy.

### Related reports

Other contents related to this tumor include ductal epithelial origin of undifferentiated carcinoma component, histiocytic origin of osteoclast-like giant cells (OGCs), more favorable prognosis than that of invasive ductal adenocarcinoma or undifferentiated carcinoma without OGCs, and excellent surgical outcomes in cases of diagnosis at early stages.

### Term explanation

OGCs are non-neoplastic, reactive, multinucleated cells and similar to those seen in giant cell tumor of bone.

### Experiences and lessons

Though an aggressive surgery for selected cases of undifferentiated carcinoma of the pancreas with OCGs may prolong survival duration, surgical role in this tumor is to be established.

### Peer review

This article is an interesting case report for a very rare tumor. To complement weakness of case report, the author added a table summarizing all cases diagnosed with this tumor.

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CASE REPORT

# Primary malignant melanoma of the esophagus: A case report

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## Abstract

Primary malignant melanoma of the esophagus (PMME) is a malignant tumor which occurs in the melanin cells of esophageal mucosal epithelial basal layer. PMME is a rare disease with an extremely poor prognosis. PMME represents only 0.1% to 0.2% of all esophageal malignant tumors. Dysphagia, retrosternal or epigastric discomfort or pain is the most frequent symptom at presentation. Retrosternal, epigastric discomfort, melena or hematemesis are the major clinical manifestations. The tumor is often located from the middle to lower thoracic esophagus. The characteristic endoscopic finding of PMME is a polypoid lesion that is usually pigmented. Immunohistochemical examination with positive results of S100 protein, HMB45 and neuron-specific enolase allow a definitive diagnosis. PMME metastasizes *via* hematogenic and lymphatic pathways. Esophagectomy is believed to be an effective approach for localized PMME. Five-year survival rates of 37% or higher have been achieved recently. Herein, we report a case of an 65-year-old female admitted for progressive difficulty in swallowing for more than 4 mo. After upper gastrointestinal endoscopy and biopsy, upper gastrointestinal series and computed tomography examination, the patient accepted radical esophagectomy, and the postoperative pathologic and immunohistochemical examination showed PMME.



**Key words:** Melanoma; Esophagus; Endoscopy; Diagnosis; Upper gastrointestinal tract

**Core tip:** We report a rare case of primary malignant melanoma of the esophagus (PMME). Although this disease is uncommon, a preoperative diagnosis of PMME is important. PMME cannot be definitely diagnosed from clinical symptoms or by X-ray barium meal examination but can be confirmed by endoscopic histological examination or a pathological report after surgical excision. The diagnosis of primary malignant melanoma should be suspected when a black or dark brown mass is observed during endoscopy. However, the endoscopic examination can complicate histological diagnosis due to poor sample quality. In the case presented here, we collected biopsies of the black surface, and the pathological report confirmed that the samples conformed to the characteristics of melanoma.

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## INTRODUCTION

Primary malignant melanoma of the esophagus (PMME) is rare tumor, representing only 0.1% to 0.2% of all esophageal malignant tumors[1]. PMME often presents as polypoid lesion and usually causes dysphagia, retrosternal or epigastric discomfort or pain. With the develop-

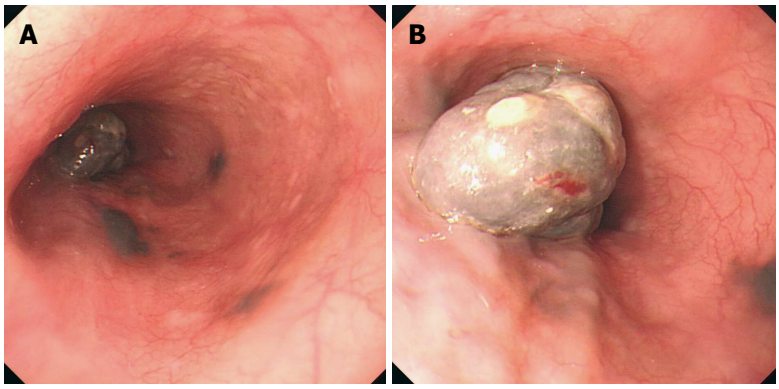


**Figure 1 Endoscopic examination findings.** A: A large (20 mm × 40 mm) tumor in the esophagus 33 cm from the incisors and 3 pigmented spots (satellites) adjacent to the large tumor 30-33 cm from the incisors; B: A partial stalk and the black uneven surface of the large tumor and the dark smooth surface of the 3 flat lesions.

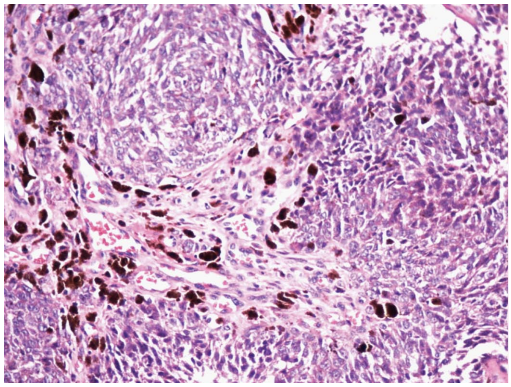

**Figure 2 Pathological findings (hematoxylin/eosin staining) from the endoscopy.** The tumor contained variously pigmented bizarre cells.

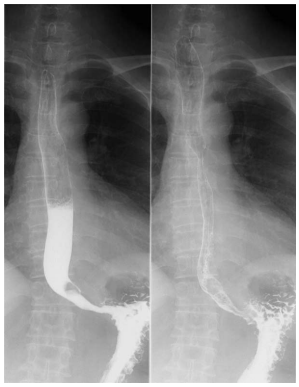

**Figure 3 Upper gastrointestinal series showing a circular filling defect in the lower esophagus.**

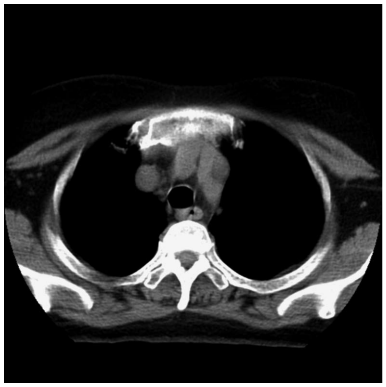

**Figure 4 Plain computed tomography scan of the cervical region, chest and upper abdomen demonstrating the thickness of the middle esophagus wall.**

ment of the endoscopic technique, the discovery rate of PMME has gradually improved in recent years. However, prognosis is not optimistic due to the high metastatic potential of PMME. Similar to most malignant tumors, early detection and treatment of PMME can improve the five-year survival rates of patients. PMME postoperative five-year survival rates have risen from 4.2% in 1989 to 37% in 2002 and may be higher now, according to current reports[2,3]. In clinical practice upper gastrointestinal series and computed tomography (CT) are also widely used to diagnose and evaluate PMME. A definitive diagnosis could be confirmed with positive results of S100 protein, HMB45 and neuron-specific enolase from pathologic and immunohistochemical detection. We recently encountered a case of PMME in endoscopy and diagnosed it by pathologic and immunohistochemical examination.

## CASE REPORT

A 65-year-old woman with progressive difficulty in swallowing for more than 4 mo recently came to our institute for an examination. The dysphagia began 4 mo prior to the examination, especially when eating solid foods, and it became more serious 2 mo prior. Upper gastrointestinal endoscopy was performed and revealed a large tumor (2 cm × 4 cm) in the esophagus 33 cm from the incisors and 3 pigmented spots (satellites) adjacent to the large tumor 30-33 cm from the incisors (Figure 1A). The large tumor had a partial stalk and a black uneven surface, and the satellites had dark smooth surfaces (Figure 1B). Upon microscopic examination, the tumor was observed to contain variously pigmented unusual cells (Figure 2). An upper gastrointestinal series showed a circular filling defect in the lower esophagus (Figure 3). A plain CT scan of the cervical region, chest and upper abdomen showed thickness of the middle esophagus wall (Figure 4). The skin examination did not reveal any evidence of melanoma. Our diagnosis was primary malignant melanoma of the esophagus, and the patient underwent an esophagectomy. Postoperative specimens confirmed the diagnosis of primary melanoma, and the surgical margin of the esophagus was free from tumors (Figures 5 and 6). Immunohistochemical staining was positive for S100

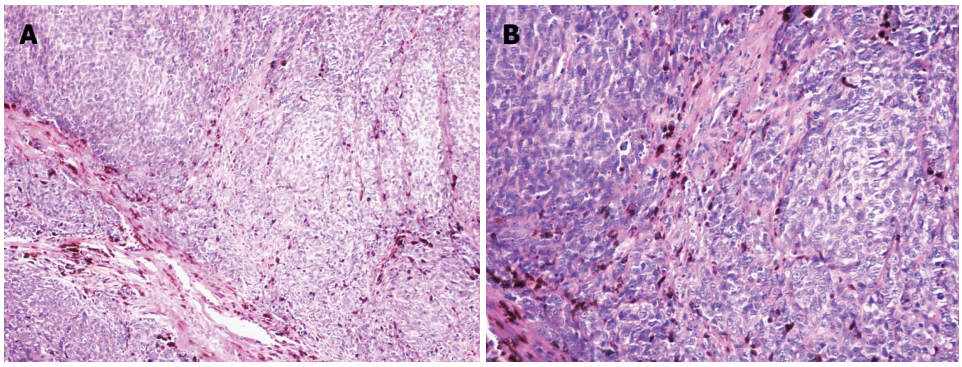


**Figure 5 Pathological findings (hematoxylin/eosin staining) from the postoperative specimens.** A: Large tumor (× 100); B: Large tumor (× 200).

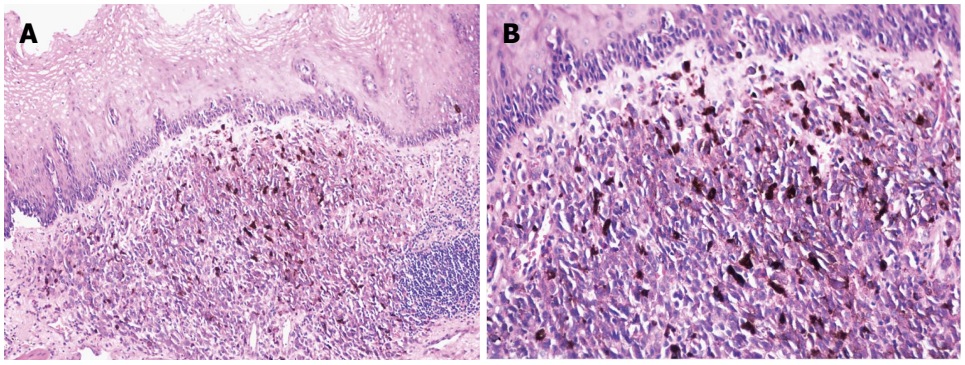


**Figure 6 Pathological findings (hematoxylin/eosin staining) from the postoperative specimens.** A: One of the satellites (× 100); B: One of the satellites (× 200).

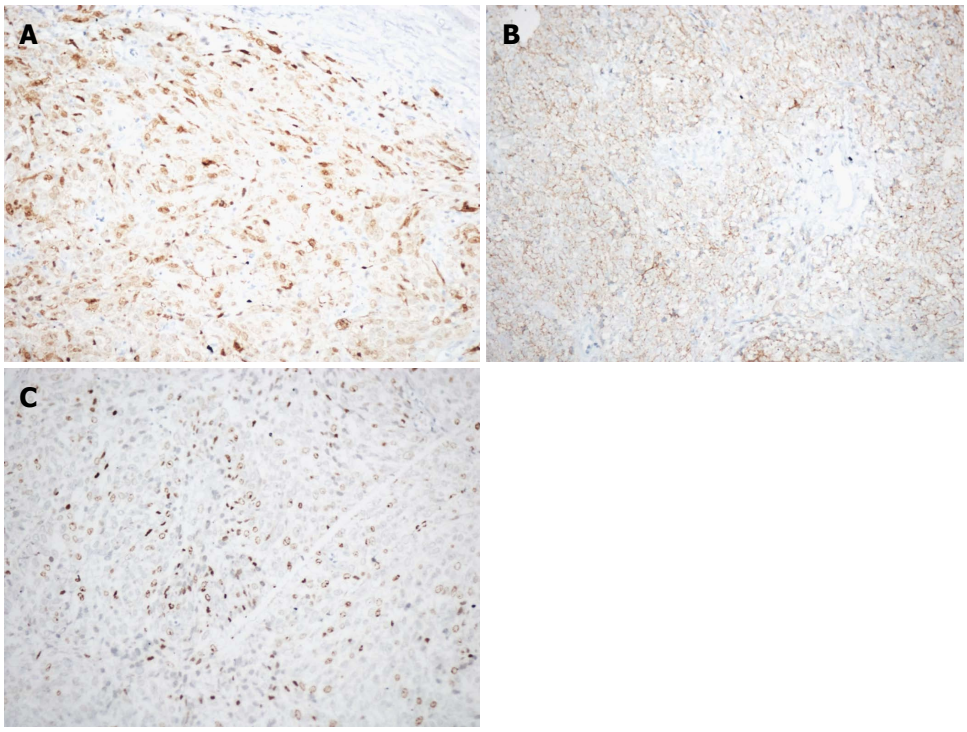


**Figure 7 Immunohistochemical staining positive for S100 (A), HMB45 (B), and Ki67 (C).**

(Figure 7A), HMB45 (Figure 7B), and Ki67 (Figure 7C). After the surgery, the patient's dysphagia was significantly improved.

## DISCUSSION

PMME cannot be definitely diagnosed from clinical symptoms or by X-ray barium meal examination. However, this disease can be confirmed by endoscopic histological examination or by pathology after surgical excision. An endoscopy may reveal friable pigmented polypoid masses that are rarely accompanied by ulcers[4]. The diagnosis of primary malignant melanoma should be suspected when a black or dark brown mass is observed

during endoscopy. However, endoscopic examinations can sometimes complicate histological diagnoses when the sample is too small, no pigment granules are present or pigment granules can not be observed because of ulceration, erosion or necrosis. When these situations occur, PMME must be differentiated from low differentiated squamous carcinoma, sarcoma, carcinosarcoma, undifferentiated carcinoma and metastatic melanoma[1]. In addition, specific pathological staining and boundary changes around the tumor also contribute to the diagnosis[5]. Positive immunohistochemical staining results for the S100 protein, HMB45 and neuron-specific enolase aid in the diagnosis of melanoma[6]. A diagnosis can also be made by observing premelanosomes in the intracytoplasm through electron microscopy[1,7].

In conclusion, we have presented a rare case of primary malignant melanoma diagnosed by endoscopic biopsy of the esophagus. We propose that the effectiveness of diagnostic guidelines or treatment will increase with an increase in the number of PMME case reports.

## COMMENTS

### *Case characteristics*

Primary malignant melanoma of the esophagus (PMME) is a rare malignant disease with a poor prognosis and accounts for 0.1% to 0.2% of all esophageal malignant tumors.

### *Clinical diagnosis*

This case involved PMME detected *via* endoscopic biopsy of the esophagus of a 65-year-old female. Dysphagia and retrosternal or epigastric discomfort or pain are the most frequent clinical symptoms.

### *Differential diagnosis*

This report summarized the characteristics of PMME, including the clinical manifestations, endoscopic features, clinical diagnosis, pathological diagnosis, treatment and prognosis

### *Laboratory diagnosis*

PMME is mainly located in the middle and lower esophagus, and the primary endoscopic manifestation is a polypoid lesion that is typically pigmented. Immunohistochemical assays positive for S100 protein, HMB45 and neuron-specific enolase could allow definitive diagnosis.

### *Treatment*

An esophagectomy is recommended for localized PMME. The five-year survival rate is almost 37%.

### *Experiences and lessons*

Gastrointestinal endoscopy and biopsy, upper gastrointestinal angiography and computed tomography examination, esophagectomy and the postoperative pathological and immunohistochemical examination contributed to the diagnosis of PMME in this case report.

### *Peer review*

This is a report about a primary melanoma of the esophagus. New standard procedures are missing *e.g.*, endoscopic ultrasound and the discussion needs clinical updates.

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### *Acknowledgments*

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1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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**Electronic journal** (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

**Patent** (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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