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REVIEW

Cytotoxic synergism of *Clostridioides difficile* toxin B with proinflammatory cytokines in subjects with inflammatory bowel diseases

Gabrio Bassotti, Alessandro Fruganti, Fabrizio Stracci, Pierfrancesco Marconi, Katia Fettucciari

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Abstract

Clostridioides difficile (*C. difficile*) is progressively colonizing humans and animals living with humans. During this process, hypervirulent strains and mutated toxin A and B of *C. difficile* (TcdA and TcdB) are originating and developing. While in healthy subjects colonization by *C. difficile* becomes a risk after the use of antibiotics that alter the microbiome, other categories of people are more susceptible to infection and at risk of relapse, such as those with inflammatory bowel disease (IBD). Recent *in vitro* studies suggest that this increased susceptibility could be due to the strong cytotoxic synergism between TcdB and proinflammatory cytokines the tumor necrosis factor-alpha and interferon-gamma (CKs). Therefore, in subjects with IBD the presence of an inflammatory state in the colon could be the driver that increases the susceptibility to *C. difficile* infection and its progression and relapses. TcdB is internalized in the cell *via* three receptors: chondroitin sulphate proteoglycan 4; poliovirus receptor-like 3; and Wnt receptor frizzled family. Chondroitin sulphate proteoglycan 4 and Wnt receptor frizzled family are involved in cell death by apoptosis or necrosis

depending on the concentration of TcdB and cell types, while poliovirus receptor-like 3 induces only necrosis. It is possible that cytokines could also induce a greater expression of receptors for TcdB that are more involved in necrosis than in apoptosis. Therefore, in subjects with IBD there are the conditions: (1) For greater susceptibility to *C. difficile* infection, such as the inflammatory state, and abnormalities of the microbiome and of the immune system; (2) for the enhancement of the cytotoxic activity of TcdB +Cks; and (3) for a greater expression of TcdB receptors stimulated by cytokines that induce cell death by necrosis rather than apoptosis. The only therapeutic approach currently possible in IBD patients is monitoring of *C. difficile* colonization for interventions aimed at reducing tumor necrosis factor-alpha and interferon-gamma levels when the infection begins. The future perspective is to generate bacteriophages against *C. difficile* for targeted therapy.

Key Words: Inflammatory bowel diseases; *Clostridioides difficile* infection; Cytokines; Tumor necrosis factor-alpha; Interferon-gamma; Necrosis; Apoptosis; Cytotoxic synergism

Core Tip: *Clostridioides difficile* is an opportunistic pathogen that is progressively increasing worldwide. Patients with inflammatory bowel diseases are particularly susceptible due to altered immunological status and the therapies adopted that favor intestinal dysbiosis and colonization by *Clostridioides difficile*. Recent *in vitro* studies also suggest that the infection might be favored by the strong cytotoxic synergism between *Clostridioides difficile* toxin B and proinflammatory cytokines, thetumor necrosis factor-alpha and interferon-gamma. The therapeutic approaches are still limited, and those presently available rely on antibiotic therapy and fecal transplantation.

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INTRODUCTION

Clostridioides difficile (*C. difficile*)[1] is a gram-positive, anaerobic, and spore-forming bacterium[2-6], responsible for the most widespread healthcare-associated infection worldwide[7-11]. In the United States every year about 500000 individuals become infected, leading to approximately 29000 deaths, and in Europe there are 124000 cases of infected individuals per year with a mortality rate ranging from 3% to 30%[7-11]. *C. difficile* infection (CDI) accounts for more than 15%-25% of all opportunistic gastrointestinal infections[7-11]. The rate of *C. difficile* colonization is about 18%-90% of healthy infants in relation to infant age[12], and 4%-15% of healthy individuals[13]. Its transmission occurs by the fecal-oral route[6,14], mainly by spores. Hospitals and community healthcare settings are an important source of infection due to the presence of *C. difficile*-infected patients[15,16]. The latter create a microenvironment highly contaminated by *C. difficile* spores extremely resistant to common strong disinfectants and radiation[17-19].

The clinical manifestations of CDI vary from asymptomatic carriage or mild self-limiting diarrhea to pseudomembranous colitis, with complications including toxic megacolon, fulminant infection and death[2-5,10]. The disease strictly depends on *C. difficile* germination and the release of three toxins[2-5,10]. The Rho-glycosylating *C. difficile* toxins A (TcdA) and B (TcdB) are major toxins that are clearly responsible for diarrhea and colitis[2-5,10]. In addition, 5%-30% of clinical *C. difficile* strains produce a binary autoprotease domain-ribosylating toxin, called the *C. difficile* binary toxin (CDT), that modifies actin[20,21].

It has previously been highlighted how the continuous progressive spread of *C. difficile* in the anthropized environment and the ability to develop more virulent strains will allow it to colonize most of the human population in the near future[22,23]. Among the subjects who will be more progressively colonized/infected with the progressively expanding *C. difficile* are those with inflammatory bowel diseases (IBD)[24-28], a category which is increasing in number in both Western and Eastern countries [29,30,31].

The progressive *C. difficile* endemic spread and the growing number of IBD subjects[25,29,32,33] are already interacting, as evidenced by studies showing how the rate of CDI cases in patients with IBD has increased by approximately four times in recent years[25]. It is therefore important to understand the

molecular and pathogenic events by which *C. difficile* colonizes and infects and how these can impact subjects with IBD characterized by a profound alteration of the microbiome, of the immune system, and of the inflammatory response.

C. DIFFICILE

CDI and clinical manifestations

CDI causes nosocomial/antibiotic-associated and community healthcare diarrhea with abdominal pain and cramps[6,10,32,33]. Colitis without pseudomembrane formation features watery diarrhea, trace blood in stool, nausea, abdominal pain, malaise, anorexia, low-grade fever, dehydration, pyrexia, and leucocytosis[6,10,32,33]. Clinical manifestations of pseudomembranous colitis consist of abdominal cramps, watery diarrhea with dehydration, hypoalbuminemia, and increased serum proteins, mucus, and inflammatory cells. Sometimes plaques (pseudomembranes) are found in the colorectal mucosa[6,10,32,33]. Fulminant colitis, observed in about 3% of CDI patients, induces serious complications such as perforation, prolonged ileus, megacolon, and death[6,10,32,33]. CDI may not be limited to the colon, and extra-colonic manifestations have been reported, including small bowel disease with the formation of pseudomembranes on the ileal mucosa, bacteremia, reactive arthritis, appendicitis, intra-abdominal abscesses, osteomyelitis, and empyema[34,35] In recent years, a significant rise in cases of fulminant colitis, which results in the development of symptoms, multiple organ failure, and increased mortality, has been associated with hypervirulent strains of *C. difficile*[6,10,32,33]. The disease strictly depends on *C. difficile* production and release of three toxins[2-5,10]. TcdA and TcdB are primarily responsible for clinical manifestations of disease[2-5,10]. However, 5%-30% of *C. difficile* clinical strains produce CDT, which contributes to disease by means of actin modification[20,21].

TcdA and TcdB: molecular structure and their receptors

TcdA and TcdB are single chain proteins, with a molecular weight of 308 kDa for TcdA and 270 kDa for TcdB[2-5,32]. Tcds show 48% sequence identity and 66% sequence similarity, where the diversity of sequence is mainly limited to the C-terminal binding domain. TcdA and TcdB are constituted by four domains, each characterized by specific biological and functional properties: (1) A glucosyltransferase N-terminal domain (GTD); (2) An autoprotease domain; (3) A pore forming and translocation domain; and (4) A C-terminal binding repetitive oligopeptides domain (CROP)[2-5,32]. The CROP domain and other amino acids outside this domain allow the binding of Tcds to the cells for subsequent internalization[2-5]. Although TcdA and TcdB CROPs display the solenoid fold, they present distinct spatial and sequential arrangements of their repeat units. This agrees with findings that suggest that both TcdA and TcdB bind to different receptors[2-5,32]; therefore, TcdA and TcdB do not follow the rule of one toxin-one receptor[2-532,36].

Two different cell surface receptors have been proposed for TcdA: rabbit sucrase isomaltase and gp96 [2-5,32,36]. Since many cells and tissues that are sensitive to TcdA do not express sucrase isomaltase and cells lacking gp96 are only partially resistant to TcdA intoxication, TcdA could bind to other receptor structures[2-5,32,36]. Three different receptors have been identified for TcdB[2-5,32,36]: Chondroitin sulphate proteoglycan 4 (CSPG4); poliovirus receptor-like 3 (PVRL3); and Wnt receptor frizzled family (FZD; FZD1, 2 and 7)[2-5,32,36]. TcdB binding to the CSPG4 receptor in HeLa and HT29 cells induces cell rounding and apoptosis at picomolar concentrations of TcdB but induces necrosis at higher concentrations of TcdB[32,36-38]. TcdB binding to the PVRL3 receptor induces necrosis at high doses (the nanomolar range) of TcdB[32,36-39]. TcdB binding to the FZD receptors induces cytopathic effects and apoptosis at picomolar concentrations of TcdB, indicating that FZD functions as an alternative receptor to CSPG4[36-40]. A further significant distinctiveness of TcdB is that it can bind to the membrane receptor with amino acid sequences that extend beyond the CROP sequences[36-40].

This description, in agreement with recent studies, indicates that TcdA and TcdB use more than one receptor for cell binding and uptake and highlights how the heterogeneity of the TcdB-bound receptors may impact on the diversity of the effects in relation to the receptor binding, concentrations of TcdB, and cell types[36-43]. It is possible that TcdB may utilize multiple receptors to broaden the selection of mammalian cells it can target[36-41]. Further, TcdB variants are highly diverse for their receptor preference, with relevant implications on colonic pathology[20,32,36,41].

The role played by CROP domains is also demonstrated by the fact that antibodies directed toward the CROP domains of TcdA and TcdB prevent uptake[2-5,36], and excess of the TcdA CROP domain compete with TcdA for cell binding[2-5,36]. However, TcdA and TcdB that lack CROP domains are still capable of internalization by the cells[2-5,36]. To understand some important aspects of the pathogenic strategy of *C. difficile* it is necessary to know what Tcd receptors are expressed on different cell types. Receptors for Tcds do not have well-defined molecular structures[20,32,36-40]; they are likely formed by a complex configuration of the polysaccharide chains that are recognized by the TcdA or TcdB binding domains with a lectin-like structure[42]. Furthermore, TcdA and TcdB have some important features of intrinsically disordered proteins[44] that allow Tcds to modify their conformation to adapt and more efficiently bind to the target structure of Tcd receptors[32,36-40]. The complex and intrinsically

disordered structural features of the Tcds allow them to bind very different cell types[2-5,32], such as the cells of surface epithelium of the human colon[43], colonocytes[45], hepatic cells[46], nervous cells [47], enteric glial cells (EGCs)[48], and cardiac cells[49].

It has been hypothesized that when *C. difficile* spores germinate into their vegetative forms and replicate that their susceptibility to the cytotoxic activity of macrophages, polymorphonucleates, and lymphocytes will increase[32,50-52]. These immune cells induce and increase the inflammatory response [3,50-53] characterized by secretion of several proinflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α)[54-58]. *C. difficile* struggles against these immune cells by means of Tcds, capable of binding to receptors with different forms of carbohydrates that give rise to distinct structures. This result is obtained by the ability of TcdB to recognize three types of receptors[36-40] and by its intrinsically disordered structure[44]. Therefore, due to accidental molecular homology, Tcds could also be cytotoxic to other cell types not present in the infection site that express one or more of the Tcd receptors (*e.g.*, endothelial, hepatic, nervous, EGCs, and cardiac cells)[32,46-49]. Furthermore, the binding capability of Tcds toward colonocytes deepens the tissue damage within and beyond the submucosa. The subsequent damage to the muscle and enteric nervous system cells creates conditions to expel *via* diarrhea the *C. difficile* vegetative forms that rapidly become spores and to start a new infection cycle. It is also possible that if the Tcd receptor domain mutates, the pathogenicity of *C. difficile* may become more severe.

Tcd uptake and internalization

Tcds, after binding to their cell membrane receptors, promote their uptake by endocytosis[2-5,32]. Tcds differ in uptake pathways. TcdB uptake is mediated by clathrin[2], while TcdA uptake is mediated by PACSIN2/syndapin-II[2]. In the endocytic vacuole the progressive pH decrease promotes a Tcd conformational change that leads to translocation across the endosomal membrane of the catalytic domain for cleavage through vacuole pore formation[2-5,32]. Then, Tcds undergo autoprocessing by the cysteine protease domain that follows the N-terminal GTD[2-5,32] in a host-factor dependent manner (*e.g.*, inositolphosphates, mainly inositol hexakisphosphate)[2-5,32], releasing the GTD into the host cell cytosol[2-5,32].

Tcd intracellular effects

The Tcds GTD, by the monoglucosylation of the catalytic site of Rho-GTPases, inhibits their activity[2-5, 32]. The monoglucosylation of Rho-GTPases induces different effects *in vitro* and *in vivo*[2-5,32]. The effects mainly documented *in vitro* are: (1) Actin condensation, rearrangement of the cytoskeleton, and disruption of focal adhesions and tight junctions[2-5,32]. These effects induce cell rounding in cultured cells (cytopathic effect)[2-6,32,48]; (2) Arrest of the cell cycle by reduction of both the expression of cyclins and the activation of cyclin-dependent kinases that together mediate progression in the different cell cycle phases[2-4,32,48]; and (3) Cell death by apoptosis or necrosis (cytotoxic effect)[2-6,32,48]. Cell death occurs in a glucosylation-dependent/glucosylation-independent way, mainly by apoptosis with caspase-dependent or caspase-independent mechanisms[2-4,32,48]. Furthermore, the cytotoxic effects are dependent on the dose of Tcds, receptors involved, and cell types[2-6,32]. In fact, TcdB at high concentrations bind to CSPG4, FZDs, or PVRL3 and induce cell death by necrosis[36-39,59]. At low concentrations TcdB binds to CSPG4 or FZDs, and cell death occurs by apoptosis[36-39,48]. However, it must be considered that the effects of Tcds could depend on cell types, likely by selective expression of Tcd receptors, and by differential expression levels of Tcd receptors[2,3,6,32].

Tcd effect in vivo

TcdA and TcdB, *in vivo*, disrupt epithelial tight junctions and induce cell death, provoking direct damage to the colonic epithelium[2-5,32]. In addition, Tcds induce an acute inflammatory response stimulating colonic epithelial cells to release proinflammatory cytokines and chemoattractants of neutrophils[2-5,32] that can induce tissue damage, modifying the barrier effect of the intestinal mucosa. A compromised intestinal barrier within the context of active inflammation subsequently leads to enhanced intestinal and vascular permeability[3,26,29,32,34]. Following the loss of a protective barrier, there is access of Tcds and/or bacteria into the lamina propria, which in turn increases intestinal inflammation[3,26,29,32,34]. TcdA primarily affects the intestinal epithelium, while TcdB has a broader cell tropism and is probably responsible for the major clinical effects of *C. difficile* due to its toxicity, which is approximately 1000 times higher than that of TcdA. Thus, TcdB represents the main virulence factor of CDI[2,3,26,29,34]. TcdB causes death in many different cell types[3,26,29,32,34] other than epithelial cells and colonic myofibroblasts, such as hepatocytes, cardiomyocytes, lung fibroblasts, immunocytes, enteric neurons, and EGCs[32,46-49].

Since immune cells that reach the replication area of the *C. difficile* vegetative form possess intrinsic motility, the molecular strategy to cause cytoskeleton alterations and cell cycle arrest is of crucial importance in order to decrease their functional ability to counteract CDI[2,3-5,6,32]. Tcds in these immune cells stimulate cytokine secretion, in particular proinflammatory cytokines such as IFN-γ and TNF-α[2,3,6,32,34] and anti-inflammatory cytokines such as IL-10[60]. Cytoskeletal disruption occurs in some cells after 30 min, representing the initial event that leads to cell rounding in most cell types *in*

vitro[32,61] with detachment of cells. Tcds *in vivo* induces both retraction of colonocytes and basal membrane cells, favoring the additional in-depth penetration of *C. difficile* and promoting an extremely inflammatory environment that causes the expulsion of *C. difficile* in the external environment by diarrhea[32,62].

Tcds and cell death

Various Tcd-infected cells, after cell-cycle arrest, die by apoptosis[2,3,5,6,32]. This could represent an important aspect of the molecular strategy of this pathogen to reduce the inflammatory response. Apoptosis is a form of cell death that occurs without inflammation and is primarily mediated by activation of the effector caspases-3 and -7, which can be triggered by a death receptor-dependent extrinsic or a mitochondria-dependent intrinsic pathway[63-65]. However, apoptosis can also be activated in a caspase-independent manner by the cleavage/activation of pro-apoptotic Bcl-2 family members and non-caspase proteases such as calpains and cathepsins[66-69]. Caspase-dependent TcdA- and TcdB-induced apoptosis has been extensively investigated[2,3,32,48,70], while there is only one study that found that TcdA can also induce caspase-independent apoptosis following cathepsin[71] and calpain activation[71].

Recently, Fettucciari *et al*[72] demonstrated that the mechanism by which TcdB induces apoptosis is much more complex than previously thought[48,70]. TcdB induced apoptosis in EGCs, a cell population of paramount importance for colonic pathophysiology, by three signaling pathways activated by calpains, caspases, and cathepsins, which are all involved in both induction and execution of apoptotic signaling[72]. Calpain activation by Ca^{2+} influx is the first pro-apoptotic event in TcdB-induced EGC apoptosis and causes caspase-3, caspase-7, and PARP activation. The latter is activated by caspases but also directly by calpains, which are responsible for the majority of apoptosis[72]. Caspase-3/caspase-7 and PARP activation is mediated also by activation of initiator caspase-8 by TcdB, and it contributes to one-third of apoptosis events[72]. Finally, cathepsin B contributes to triggering the pro-apoptotic signal and to one-third of apoptosis events by a caspase-independent manner. It appears to control the levels of caspase-3 and caspase-7 active fragments, highlighting the complex interaction between these cysteine protease families activated during TcdB-induced apoptosis[72].

Recently, we also demonstrated that pro-inflammatory cytokines, TNF-α plus IFN-γ (CKs) strongly increased apoptosis induced by TcdB, by an enhanced activation of the three pro-apoptotic pathways induced also by TcdB alone activated by calpains, caspases, and cathepsins, which are involved in both induction and execution of apoptotic signaling[72]. However, two important differences between TcdB- and TcdB + CKs-induced apoptosis are: (1) Apoptotic signaling activation by TcdB + CKs is enriched by TNF-α-induced NF-kB signaling, inhibition of JNK activation, and activation of AKT[72]; and (2) Apoptosis induced by TcdB + CKs increased strongly in the time course, with more than 60% of the cells undergoing apoptosis at 72 h, while apoptosis induced by TcdB increased slowly, with only about 18% of cells undergoing apoptosis at 72 h[72].

This capability of TcdB to trigger three different cell death pathways represents an extremely important *C. difficile* strategy[72] to overcome the possible intrinsic resistance of a cell type to one or two of the three apoptotic pathways. In fact, as reported above, Tcds are able of cause cell death in different non-immune[32,46-49] and immune cell types[2,3-32,73,74].

A further strategy adopted by *C. difficile* is its ability to induce different types of cell death that can lead to different consequences in the *C. difficile* pathogenesis[2-4,6,32]. In fact, TcdB causes cell death by both apoptosis and necrosis[2,3,6,32,75] that is dependent on the TcdB concentration and by the TcdB receptor expressed by the target cells[20,32,36-38]. TcdB at lower concentrations and binding to the CSPG4 or FZDs receptors[20,36-40] induces apoptosis in a glucosylation-dependent manner[2-6,32], while at higher concentrations (100 pM or above) causes cell death by necrosis[20,36-43], which does not require either the autoprocessing or glucosyltransferase activities of the toxin[2-6,75]. Necrosis is an early process occurring after 2-4 h of intoxication and is found in both cell culture and colon explant models[2,3,5,-6,75]. Necrosis is characterized by quick ATP depletion, early loss of plasma membrane permeability and cellular leakage, and chromatin condensation without caspase-3 and caspase-7 activation[2,3,6,32,75].

TcdB causes necrosis through activation of a strong production of reactive oxygen species (ROS) by assembly of the NADPH oxidase complex on endosomes[2-5,75]. High levels of ROS trigger necrosis by DNA damage, lipid peroxidation, protein oxidation, and/or mitochondrial dysfunction[2,3,6,32,75]. It has been suggested that pore formation in the endocytic vacuoles is important for the glucosylation-independent necrotic cell death caused by TcdB. Indeed, a TcdB mutant, defective in pore formation, does not induce necrosis even at high nanomolar concentrations[2,4,6,75]. Unlike TcdB, TcdA does not trigger ROS production through the NADPH oxidase complex and causes glucosylation-dependent apoptosis at all concentrations tested[2-6]. The ability of TcdB, but not of TcdA, to cause necrosis may explain why a wild-type ($TcdA^+TcdB^+$) epidemic strain and an isogenic $TcdA^-TcdB^+$ mutant in animal infection models provoke considerably more damage to colon tissue than an isogenic $TcdA^+TcdB^-$ mutant strain[2-6]. The glucosylation-independent mechanism of TcdB might play a similar role in the context of human disease; TcdB-induced necrosis likely contributes to the extensive gut damage observed in patients with severe forms of CDI[2-6].

TcdB induces an early cell death defined pyknotic cell death, characterized by chromatin condensation, cell cycle arrest, and ballooning of the nuclear envelope that is both glucosyltransferase domain-dependent and -independent[2,3,6,75] and occurs at concentrations 5000 times greater than necessary for Rho protein glucosylation and ROS production[2-5,75].

It has also been reported that TcdA and TcdB trigger pyrin inflammasome activation in an apoptosis-associated speck-like protein with a caspase recruitment domain-dependent manner, causing release of IL-1β[2-6]. In particular, TcdB-induced inflammasome activation triggers a type of cell death defined as "pyroptosis," characterized by cell swelling and lysis with gross release of cellular content and inflammatory cytokines like IL-1β through pore formation in target cells caused by caspase-1-dependent activation of gasdermin D, which induces strong inflammation[2-6].

Most importantly, EGCs intoxicated with low doses of Tcds might revert to their normal functions after a brief cell cycle arrest[32,48,76], while EGCs that survive apoptotic activity of TcdB become senescent as a TcdB-mediated survival response to stressful stimulus[32,48,76]. This ability of cells surviving the cytotoxic activity of Tcds to become senescent may affect functionality of EGCs, intestinal neurons, and myocytes, contributing to altered bowel motility[32,77]. The acquisition of a senescence status by these cell types could have critical outcomes in the subsequent development of post-infectious irritable bowel syndrome and stimulation of preneoplastic cells[32,77].

Thus, we can postulate that when healing after an acute CDI patients can later have some important consequences such as a decrease in EGC number and impairment and alterations in EGC functionality [32]. After CDI, the structural and functional defects caused by the Tcds might be long-lasting in a significant percentage of patients and trigger low-grade inflammation and persistent dysmicrobism[25]. This implies that residual *C. difficile* bacteria that remain when healing after an acute CDI may benefit from this condition and provoke relapses. The latter might be due to cytotoxic synergism with inflammatory cytokines that can occur even after months without any evident cause[78]. Therefore, it is possible that *C. difficile* changes the large bowel environment to remain for a long time and favor easier relapses. This means a continual expansion of *C. difficile* carriers in the large bowel environment, with induction of an irritable bowel syndrome-like condition and with recurrences due to a latent or fluctuating inflammatory condition as in IBD subjects.

All this emphasizes the complex molecular strategy of *C. difficile* based on the cytotoxic synergism with some components of the inflammatory response as IFN-γ and TNF-α, which potentiate cytotoxicity of TcdB[48,72]. Therefore, it is conceivable that IFN-γ and TNF-α behave as drivers of the infection from the beginning of the infection, amplifying apoptosis induced by low doses of Tcds, and opening the way to infection progression[26,32,34,48,72].

Therefore, it is likely that antibiotic treatment, other than provoking dysmicrobism, by means of bacteriolytic activity builds an inflamed environment in the large bowel by release of bacterial factors from killed bacteria. Additionally, whatever inflammatory environment induced in the absence of antibiotic treatment could also help CDI in subjects with obesity or other pathologies accompanied by an inflammatory state[28].

THE CONDITIONS THAT FAVOR THE ENDEMIC SPREAD OF *C. DIFFICILE* IN PEOPLE WITH IBD

An endemic spread of *C. difficile* in IBD patients is favored by:

(1) The extreme resistance of the spores to the external environment and the mechanism of spore germination[17-19,79].

C. difficile spores are extremely resistant to strong disinfectants and radiation[17-19]. The mechanism of germination is both complicated and distinctive compared to that of other microorganisms[79], due to the peculiar Tcd interactions with the host and to cellular microbial factors that facilitate the colonization and successive infection and relapses[79-83]. Moreover, *C. difficile* spores can also adhere to gut epithelial cells and penetrate them *via* a process of macropinocytosis-like endocytosis[84,85]. This macropinocytosis-like endocytosis is dependent on Fr-95B_1 and Vn-$a_v\beta_1$ integrin and on the spore-surface collagen-like BclA3 exosporium protein[84,85]. In an *in vivo* model in mice, it has been shown that the entry of spores into intestinal epithelial cells in a dormant but reactivable state contributes to the recurrence of CDI[84,85].

(2) Progressive colonization of humans by the *C. difficile* spores depends on *C. difficile* to wait for the appropriate conditions that favor transition to the vegetative form capable of inducing infection and the clinical manifestations. Colonization is due to *C. difficile* transmission *via* the fecal-oral route, with the spores that traverse the acidic pH of the stomach to colonize the large bowel, where they remain inactive until appropriate conditions favor the passage to the vegetative form[83]. The conditions that favor intestinal germination of *C. difficile* spores are an increase of primary bile acids, butyrate, disaccharide, and trehalose and other substances produced by some bacterial species taxonomically identified in a perturbed microbiome that favors *C. difficile* overgrowth *vs* other pathogens and a reduction of secondary bile acids[86-91]. Of interest, these conditions have been described in patients with IBD[92,93]. In fact, *C. difficile* colonizes and infects the colon following antibiotic-immunosup-

pressant-induced gut dysbiosis[94-101]. The dysbiosis also depends on different factors such as age, type of drug used, administration of proton-pump inhibitors, types of foods, physical environment, the genetic and immune system of the individual, and concomitant pathologies (*e.g.*, diabetes, obesity, autoimmune and allergic diseases, IBD)[94-101]. These predisposing factors have progressively broadened the range of subjects susceptible to colonization/infection by *C. difficile*[94-101]. In turn, *C. difficile* colonization causes gut flora perturbations that increase dysmicrobism and inflammation, promoting CDI and CDI relapses[94-101]. Moreover, changes in normal gut microflora after a first CDI could predispose individuals to recurrent CDIs, and the protracted antimicrobic therapy for *C. difficile* can cause, in a gut microbiota already modified, further and persistent dysbiosis and inflammation.

Although following primary CDI episodes the bacterial taxa restore with time, in some subjects some taxa may not recover fully and maintain a decreased resistance to colonization. This in turn promotes the subsequent growth of pathogens (including *C. difficile*), altering the composition of the gut microbiome. The frequent use of antibiotics to treat *C. difficile* increases the pool of antibiotic-resistant genes in the gut microbiome, thereby favoring recurrent CDIs[96-103]. Although antibiotic exposure, hospitalization, advanced age, and immunocompromised status increase the risk for disease, community-acquired infections in otherwise healthy young and adults not previously exposed to antibiotics are not infrequent[96-103].

The favorable conditions for *C. difficile* colonization are more widespread in "developed countries" due to the increase in antibiotic therapies[15] in all ages and changes in microbiota for various external factors[104]. Thus, a progressive increase of CDI and CDI-related deaths (at present in the range of 5%-30% with primary infection)[16] is foreseeable, with a more progressive rise of death rates following CDI relapses[105,106].

(3) Persistent dysmicrobism due to the use of antibiotics, immunosuppressive drugs, and most important a colonic environment characterized by waves of inflammatory response with a continuously active basal level. Persistent dysmicrobism enables the overgrowth of several intestinal pathogens, including *C. difficile. S*ome particularities of *C. difficile* favor its growth in an altered environment characterized by low-grade inflammation[15,80-82,90,98-101,104], such as in a subject with IBD[24-31]. For primary CDI, the changes in gut microbial flora that favor overgrowth of *C. difficile* over other various intestinal pathogens (*e.g., C. perfringens*) are crucial, even though the role of the gut flora in regulating *C. difficile* is more complex than previously hypothesized. Indeed, in preventing *C. difficile* colonization, disease, and recurrence, the maintenance of enough density of the species creating an environment hostile to *C. difficile* expansion by means of both changes of biomass and composition rather than the simple reduction of some taxonomic groups plays a key role[9,90,94-101]. Furthermore, dysmicrobism depends primary on the factors and pathologies reported above (*e.g.,* IBD)[9,90,94-101].

(4) Continued emergence of new *C. difficile* strains that are more hypervirulent or multidrug-resistant (*e.g.,* ribotypes 015, 027, 078 or 176), many of which produce the binary toxin CDT[20,51,91,107,108].

(5) Production of variant Tcds by some *C. difficile* strains[51,80,91,99,102]. The expansion of CDI could be enhanced by *C. difficile* strains that produce Tcd variants[107-111]. In fact, many of these are hypervirulent and release the binary toxin CTD, which is linked with enhanced morbidity and mortality [,107-111]. CTD stimulates the formation of long cellular filaments, which become anchor points for other *C. difficile* to epithelial cells, potentiating the infection[20,51,91,107,108]. The Tcd variants are also greatly different for enzymatic activity, immunogenicity, and their receptor preference, with important implications on colonic pathology[51,91,107,108].

(6) The complex equipment of surface antigens of *C. difficile* are flagella, fimbriae, pili, cell wall proteins, and biofilm, which act as colonization factors or mediate innate immune responses that can play a key role in the persistence of *C. difficile*[2,3,6,10,32] and induce proinflammatory cytokines such as TNF-α and IFN-γ[2-6].

IMPACT OF THE LATEST KNOWLEDGE ON THE MOLECULAR PATHOGENESIS OF *C. DIFFICILE* TOXINS ON COLONIZATION/INFECTION IN INDIVIDUALS WITH IBD

The receptors for TcdA and TcdB

TcdB is the most involved in CDI due to its presence in most toxic strains and its degree of pathogenicity is 1000 times greater than that of TcdA[2-6]. The functions of the TcdB receptors, CSPG4, PVRL3, and FZD, are summarized above. The variants of TcdB are further diversified for their receptor preference with relevant implications in the pathology of CDI[32,51,91,107,108] by the ability to bind extremely different cell types[2,3,6,32], including human colon epithelium cells[43], nerve cells[47], EGCs[48], neurons, liver cells, and heart cells[49].

What are the elements that can therefore impact subjects with IBD as a consequence of the heterogeneity of Tcds and of their receptors on the various cell types? The intestinal mucosa of IBD subjects is altered by inflammation resulting from the immune response and dysmicrobism. Although direct data on how this altered colonic environment may modify the expression of the various receptors for TcdB are not available, it is likely that this could occur, as suggested by *in vitro* data. Therefore, it is possible that conditions whereby a differential expression of TcdB receptors are expressed on colonic epithelial

cells favoring primarily the necrotic effects of TcdB could arise, characterizing the trend of infection. Moreover, even the inflammatory immune cells that try to fight infections could be induced to express receptors that lead to their cell death by necrosis. Thus, in subjects with IBD an initial CDI could quickly become serious due to a strong inflammatory response that favors the expression of receptors for the TcdB that lead to death mainly by necrosis. This would profoundly change the environment already altered by necrosis to favor further relapses.

It is therefore possible that the progression and severity of CDI in subjects with IBD depends on an inflammatory environment inducing the prevalent expression of receptors that favor cell necrosis. Indeed, it has been demonstrated that expression of CSPG4 is increased by inflammatory conditions such as that induced by TNF-α and lipopolysaccharides[112,113].

Synergy of the inflammatory response and TcdB enhances the toxicity of TcdB

CKs release potentiates apoptosis of EGCs treated with low doses of TcdB[48,72]. This phenomenon is relevant with profound implications *in vivo*[26,,32,34,48,72], especially in subjects with IBD[29].

First, the enhancement of apoptosis induced by the synergism between TcdB +CKs also occurs when CKs are given to EGCs 18 h before TcdB[48]. Therefore, in an already inflamed environment, as soon as *C. difficile* begins to produce Tcds, cytotoxicity is immediately increased by the presence of pre-existing cytokines, paving the way for the progression of CDI.

Second, the cytotoxic synergism between TcdB and CKs is triggered even 3 d after infection with TcdB[48,72], implying that even if at the beginning of the infection there is no significant inflammatory response, the enhancement of cytotoxicity by cytokines can occur later.

Third, the cytotoxic synergism between TcdB + CKs at 24 h is mainly characterized by death by apoptosis[48,72], while cells surviving in the following days progressively die by apoptosis/necrosis [72]. This in contrast to the cells treated only with TcdB that die by apoptosis at 24 h, and in the following days there is only a slight increase in cell death by this mechanism[72].

Fourth, cell death induced by TcdB + CKs is characterized by the activation of three apoptotic pathways[72], with a primary role played by calpains and subsequently cathepsin B activation, which either directly or converging on the effector caspases (caspases-3 and -7) lead to cell death, bypassing any anti-apoptotic barrier in the first 24 h[72]. Thereafter, a process of amplification of the cell death process begins in the cells that have resisted apoptosis, with consequent death by apoptosis and necrosis [72]. It is therefore clear how the cytotoxic synergism between TcdB + CKs finds in subjects with IBD a particularly favorable environment that will favor CDI, characterized by a strong cell death response by necrosis, with an increase in mortality and major incidence of CDI relapses. These are due to the fact that, although partially restored with antibiotic therapy, the intestinal environment remains very susceptible to further CDI relapses for the characteristics of necrotic cell damage and the persistence of the inflammatory response.

Therefore, in subjects with IBD a circuit of progressive cell damage can be activated, which feeds on itself based on the elements that characterize the first event of colonization/infection by *C. difficile*, *i.e.* the presence of an inflammatory state that enhances the cytotoxic synergism of TcdB + CKs, with induction of apoptosis and necrosis that in turn could lead to increased expression of receptors for TcdB. The latter, based on TcdB receptors involved and TcdB concentration, will promote cell death by apoptosis and/or necrosis. Cell death causes deeper tissue damage with a progressive increase in this loop that can only be stopped with antibiotic therapy, which adds an additional level of inflammatory response. Once the infection is resolved, the environment is even more susceptible to relapses.

It is clear that if CDI occurs in a subject with IBD the clinical picture can progress to an more acute form for the following reasons: (1) The expression of high levels of receptors for TcdB that induce cell death by necrosis; and (2) Higher levels of cytokines that enhance the development of receptors for TcdB, inducing mainly necrosis. The final result is enhancement of cell death by apoptosis/necrosis.

The above considerations could explain the differences in colonic environment during CDI in individuals with IBD and therefore the susceptibility to one or more relapses[29] (Figure 1).

Perspectives for future approaches to CDI treatment

Based on some peculiarities of the pathogenesis mechanisms of *C. difficile* in subjects with IBD we can try to adopt new strategies to counteract CDI in these subjects. However, we need to bear in mind that to date efficient strategies are strongly limited for the following reasons:

(1) It is not possible to block the endemic spread of *C. difficile* and to eradicate it in hospitals or nursing homes, which represent one of the most contaminated environments and the greatest cause of spread.

(2) Antibiotics or treatments preventing the development of conditions that favor relapses are not available.

(3) Fecal transplantation, although effective, is still a limited therapeutic.

(4) Immunotherapy with monoclonal antibodies to TcdA and TcdB has yielded very limited results.

(5) Vaccination against TcdA and TcdB did not yield significant clinical and eradication results for *C. difficile*.

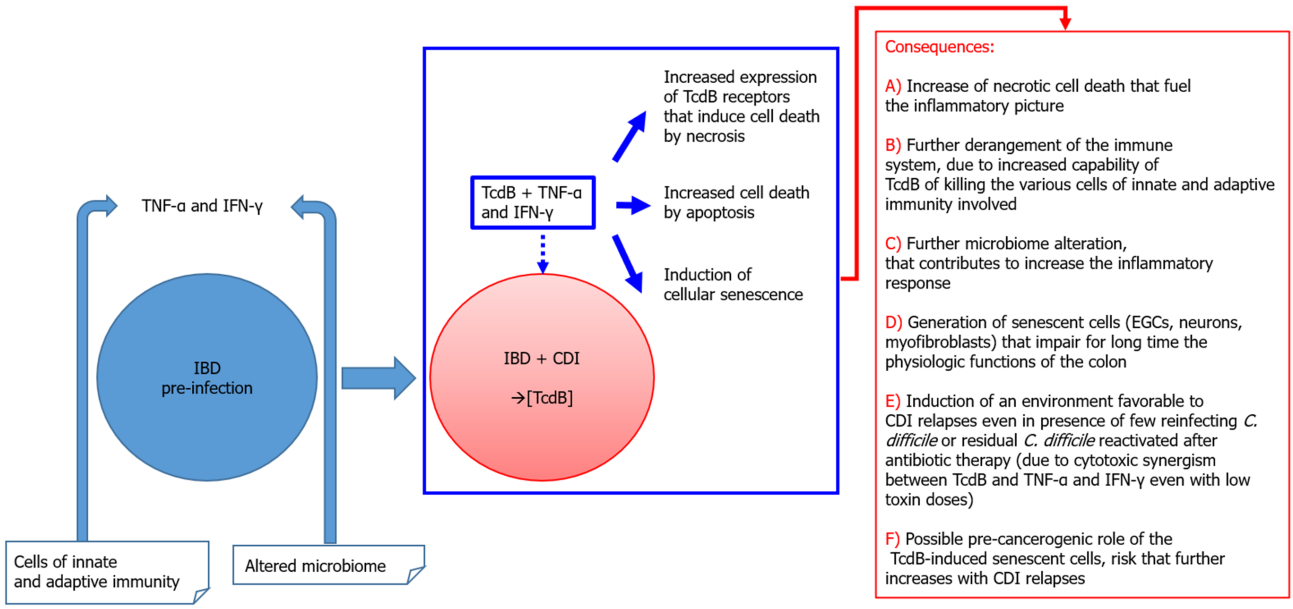

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Figure 1 Causes of increased susceptibility and increased cytotoxic effect of *Clostridioides difficile* in subjects with inflammatory bowel disease. CDI: *Clostridioides difficile* infection; *C. difficile*: *Clostridioides difficile*; EGC: Enteric glial cells; IBD: Inflammatory bowel disease; IFN-γ: Interferon-gamma; TcdB: Toxin B of *Clostridioides difficile*; TNF-α: Tumor necrosis factor-alpha.

Therefore, assuming that the spread of *C. difficile* cannot be stopped and that subjects with IBD are more at risk of contracting CDI, based on the most recent knowledge on the molecular pathogenesis of *C. difficile*, which methodology of interventions can we try to develop? Here we propose several approaches: (1) Monitor subjects with IBD for *C. difficile* with greater frequency to identify the onset of the active phase of the disease as early as possible; (2) In subjects with active IBD, the presence of colonization by *C. difficile* represents a serious risk and requires a strong reduction in the inflammatory response before the infection begins or spreads; (3) In subjects already colonized with *C. difficile* and quiescent IBD, continue microbiological monitoring by evaluating the extent of colonization over time. In cases of IBD exacerbation intervene to reduce TNF-α and IFN-γ levels using appropriate targeting drugs; (4) Due to the limited availability of specific antibiotics for CDI and the continuous emergence of antibiotic resistance, the research for alternative methods is under way. For instance, pangenomic analysis of this bacterium has revealed specific drug targets toward the core genome of *C. difficile*[114]. This, in the next future, will likely pave the road for more targeted therapeutic approaches; and (5) In high-risk subjects it would be important to develop selective prophylaxis and therapy based on highly specific bacteriophages for *C. difficile*. Indeed, this is becoming a hot topic due to the impending problem of multidrug-resistant bacteria. Since it is presently possible to analyze intestinal phages, this could represent a tool of paramount importance in the future development of phage therapy[115,116]. Of interest, the feasibility of combination phage therapy to treat infections associated with IBD was recently demonstrated[117].

CONCLUSION

The increasing worldwide spread of CDI represents a serious health problem, enforced by resistance of many bacterial strains to antibiotic therapy. This is particularly worrisome for some patient groups, such as elderly institutionalized subjects, immunocompromised subjects, and subjects with IBD[118]. The latter are particularly at risk due to the basal impaired immunological status and the frequent use of antibiotics and immunosuppressant agents for treatment. It is therefore of paramount importance to understand the mechanisms favoring CDI in these patients in order to develop more targeted and effective therapeutic strategies to limit/abolish relapses, morbidity, and mortality due to this infection. It is most important to have understood that *C. difficile* to efficient colonize humans, uses key inflammatory response elements such as proinflammatory cytokines, TNF-α and IFN-γ, that favor cell death by necrosis which increases inflammation and the expression of TcdB receptors that in turn promote further necrotic cell death. It is absolutely mandatory to find prophylactic and therapeutic methodologies to antagonize this synergism between *C. difficile* and the inflammatory response. This is important to protect patients with IBD and to prevent other at-risk populations.

FOOTNOTES

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REVIEW

Immune and metabolic cross-links in the pathogenesis of comorbid non-alcoholic fatty liver disease

Stanislav Kotlyarov

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Abstract

In recent years, there has been a steady growth of interest in non-alcoholic fatty liver disease (NAFLD), which is associated with negative epidemiological data on the prevalence of the disease and its clinical significance. NAFLD is closely related to the metabolic syndrome and these relationships are the subject of active research. A growing body of evidence shows cross-linkages between metabolic abnormalities and the innate immune system in the development and progression of NAFLD. These links are bidirectional and largely still unclear, but a better understanding of them will improve the quality of diagnosis and management of patients. In addition, lipid metabolic disorders and the innate immune system link NAFLD with other diseases, such as atherosclerosis, which is of great clinical importance.

Key Words: Non-alcoholic fatty liver disease; Metabolism; Lipid metabolism; Lipid; Fat; Innate immune system; Pathogenesis

Core Tip: Non-alcoholic fatty liver disease (NAFLD) is an important medical and social problem. The development of NAFLD is closely related to the metabolic syndrome, which further increases attention to the problem. The pathogenesis of NAFLD is complex and involves closely intertwined metabolic and immune mechanisms, a better understanding of which will improve the effectiveness of measures to prevent and treat the disease. Lipid metabolism has multiple connections with the innate immune system, in which various liver cells are involved.

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INTRODUCTION

Interest in non-alcoholic fatty liver disease (NAFLD) has increased significantly in recent years, due to an increasing number of reports on its high prevalence and clinical significance[1]. Epidemiologic data show that the prevalence of NAFLD in the adult population ranges from 17% to 46%, but the data vary by region and depend on age, sex, and several other characteristics[2]. These negative epidemiologic findings are thought to be related to the high prevalence of metabolic diseases, such as obesity and diabetes mellitus, which is due to the effects of low physical activity and poor diet[3]. The links of NAFLD with the metabolic syndrome are attracting increasing attention from clinicians. Dyslipidemia, obesity, insulin resistance, and diabetes are important features of the metabolic syndrome and are closely related to NAFLD[4-6]. Indeed, the prevalence of NAFLD among obese adults is 80%-90%, approximately 30%-50% in patients with diabetes, and up to 90% in patients with hyperlipidemia[7].

Another problem associated with NAFLD is that the disease is often not diagnosed in a timely manner, as patients do not seek medical care for a long time. Most patients are asymptomatic or the symptoms are nonspecific, and patients may not pay enough attention to them. In addition, these patients often have comorbidities, the clinical picture of which may be more pronounced and of greater concern to patients. Atherosclerotic cardiovascular diseases are common in these patients, significantly affecting quality of life and prognosis[8-10]. It is important to note that accurate diagnosis of NAFLD is currently associated with a number of difficulties, primarily, the limited availability of modern diagnostic tools in the primary care setting. Thus, NAFLD is currently a growing burden on patients and healthcare systems.

NAFLD includes two morphological forms, non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH)[11,12]. At the same time, the diagnosis of NAFLD assumes the exclusion of secondary causes and significant alcohol consumption.

NAFLD is characterized by excessive fat accumulation in the liver, but the pathophysiology of this disorder involves complex mechanisms. According to the "two-hit hypothesis" model proposed in 1998 by Day *et al*[13], the "first hit" involves lipid accumulation in hepatocytes and development of steatosis, which is associated with the negative impact of obesity, type 2 diabetes, dyslipidemia and other metabolic risk factors on the liver[13-15]. The "second hit" leads to damage to the hepatocellular system and liver inflammation and is associated with the effects of oxidative stress and proinflammatory cytokines[13]. A growing body of evidence suggests that NAFLD develops as a result of a complex chain of events, many of whose links are cross-linked, consistent with the newly proposed "multiple parallel-hit" concept. Thus, insulin resistance, de novo lipogenesis, local and systemic inflammation, disorders in the structure of the gut microbiota, and oxidative stress play an important role in the pathophysiology of NAFLD and have crosslinks that involve different cells (Figure 1)[16]. Recent advances in the study of the mechanisms that contribute to the development and progression of NAFLD have led to a better understanding of the complex interplay between environmental factors, the gut microbiota, metabolism, and the innate immune system, which include both intrahepatic and extrahepatic events[17].

MOLECULAR MECHANISMS INVOLVED IN NAFLD PROGRESSION

The significance of metabolic disorders in the pathogenesis of NAFLD

The results of studies suggest that NAFLD exhibits a close bidirectional relationship with the metabolic syndrome[18]. The development of the metabolic syndrome may precede NAFLD or be a consequence of it[19,20]. NAFLD significantly increases the risk of metabolic syndrome and may also be considered an independent risk factor for some cardiovascular diseases[21-23]. Given that NAFLD is often combined with metabolic diseases such as obesity, type 2 diabetes, hyperlipidemia, and hypertension, it may have negative prognostic implications[24,25]. Thus, an overweight person is a typical NAFLD patient phenotype[26,27]. Moreover, body mass index and NAFLD show a strong correlation[27,28]. Interestingly, NAFLD also occurs in non-obese individuals, with the majority of these findings occurring in Asian countries, although they have been described worldwide[29-32]. Despite the phenotypic differences, NAFLD patients who were not obese had similar severity of histologic liver damage[33]. At the same time, NAFLD patients without obesity had a higher degree of fibrosis[34-37].

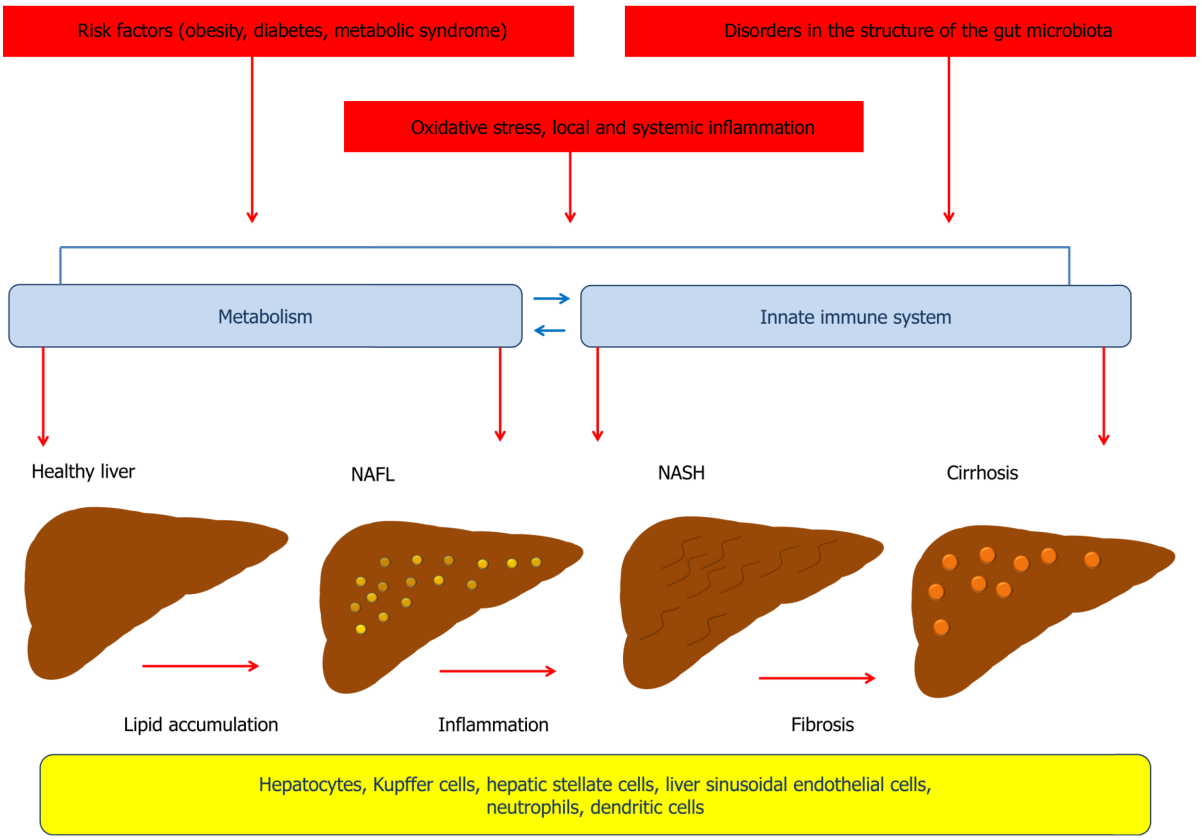

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Figure 1 Risk factors and links of non-alcoholic fatty liver disease pathogenesis. NAFL: Non-alcoholic fatty liver; NASH: Non-alcoholic steatohepatitis.

A key histological characteristic of NAFLD is the cellular accumulation of triglyceride (triacylglycerides, TAGs) containing lipid droplets[38-40]. TAG biosynthesis is carried out using fatty acids, which may enter the cells from the blood or be formed by de novo lipogenesis and endocytotic recycling of lipoprotein remnants[40,41]. In most cases, the main source of fatty acids used for TAG formation is absorption from the blood[41,42]. Interestingly, some data suggest that TAG accumulation per se is not harmful to hepatocytes and can even be considered as a certain protective mechanism against lipotoxicity induced by free fatty acids[43]. This is supported by the data that an excess of free fatty acids in nonfat cells can lead to their dysfunction and apoptotic death[44]. Moreover, levels of free fatty acids in the blood are related to the severity of NAFLD, with saturated fatty acids being more hepatotoxic than unsaturated fatty acids[45]. Thus, free fatty acids are important mediators of excessive lipid accumulation in the liver.

Studies have shown that monounsaturated fatty acids such as oleic or palmitoleic acids are less toxic than saturated fatty acids such as palmitic or stearic acids[46,47]. Long-chain saturated palmitate induces apoptosis in Chinese hamster ovary cells through a mechanism involving reactive oxygen species (ROS) and ceramide formation, which can enhance palmitate-induced apoptosis signals[43]. In turn, unsaturated fatty acids prevent palmitate-induced apoptosis by directing palmitate to triglyceride pools and removing them from pathways leading to apoptosis[43]. In doing so, reducing the ability of cells to synthesize triglycerides contributes to lipotoxicity[43]. The mechanism of this action may be related to the fact that palmitate is poorly incorporated into cellular triglyceride pools in the absence of additional signals, but the presence of unsaturated fatty acids can help direct palmitate toward triglyceride storage, thereby excluding palmitate from apoptotic pathways. Moreover, unsaturated fatty acids, which come both as additives to the medium, such as the addition of oleate, and as a result of the action of desaturase (*e.g.*, stearoyl-CoA desaturase), demonstrate this action[43]. Stearoyl-CoA desaturase-1 (SCD), known as fatty acid desaturase, is an enzyme that is expressed in the liver and is involved in the biosynthesis of monounsaturated fatty acids, primarily oleate and palmitoleate from corresponding saturated fatty acids. Decreased expression and activity of SCD1, leads to the intake of excessive amounts of saturated fatty acids, increasing their lipotoxic effects and the development of steatohepatitis and fibrosis[48,49]. Indeed, oleic acid has been shown to be more steatogenic but has less apoptotic effects than palmitic acid in hepatocyte cell cultures[50].

Increased fat in the liver correlates directly with changes in plasma saturated fatty acids and inversely with polyunsaturated fatty acids (PUFAs)[51]. Saturated fatty acids markedly induce fat deposition in

the liver and serum ceramides, whereas dietary PUFAs prevent fat accumulation in the liver and reduce ceramides and hyperlipidemia with excess energy intake in overweight people[51]. Higher concentrations of total ω-6 PUFAs and serum linoleic acid have been shown to be associated with lower odds of developing NAFLD in the future[52]. Meanwhile, ω-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may have a protective effect on the liver by reducing insulin resistance, reducing inflammation, and inhibiting apoptosis of hepatocytes[53].

These and other data allow us to expand our views on the features of metabolic processes in NAFLD, as well as NAFLD comorbid relationships. It has been shown that in NAFLD, regardless of the presence or absence of obesity, there is a high risk of coronary atherosclerosis, which contributes to the clinical picture[54]. It is widely known that NAFLD is associated with the development of atherosclerosis[55-57]. Moreover, NAFLD is associated with an increased risk of cardiovascular disease beyond that due to established risk factors[57]. Moreover, cardiovascular disease is the main cause of death in NAFLD patients[55].

NAFLD patients often have dyslipidemia along with other features of the metabolic syndrome. NAFLD patients have significantly elevated levels of oxidized low-density lipoprotein (LDL), and a significant association has been shown between LDL levels and the prevalence of NAFLD[58,59]. Elevated LDL levels within the normal range were associated with an increased risk of NAFLD[59]. In addition, there are important differences in LDL and high-density lipoprotein (HDL) subfractions in NAFLD patients. Liver fat has been shown to correlate more strongly with circulating HDL2 cholesterol and the ratio of HDL2 to HDL3 cholesterol than with total HDL cholesterol[60]. Patients with NASH had an increased number of small, dense LDL3 and LDL4 particles[61]. These changes may contribute to the increased risk of atherosclerosis and cardiovascular disease in these patients.

THE IMPORTANCE OF INNATE IMMUNITY IN THE PATHOGENESIS OF NAFLD

A growing body of evidence is increasing the understanding of the importance of the innate immune system in the development of NAFLD (Figure 2). The innate immune cells, which include Kupffer cells, neutrophils, dendritic cells (DCs), and natural killer (NK) cells, play an important role in the pathogenesis of NAFLD. Kupffer cells, which constitute 80% to 90% of the total macrophage population, are under physiological conditions a long-lived and self-renewing population[62]. Due to their location, they are central to innate immunity and are responsible for the rapid removal of exogenous particles such as lipopolysaccharide (LPS)[63-65]. Like other macrophages, Kupffer cells are also capable of detecting endogenous molecular signals resulting from homeostasis disruption[62].

Steatohepatitis is characterized by marked enlargement and aggregation of Kupffer cells in perivenular regions, with scattered large fat vacuoles found within Kupffer cells[66]. The contribution of macrophages originating from blood monocytes to this cell pool is not entirely clear, as there is currently no marker to distinguish them from resident macrophages[67].

Kupffer cells, which are resident macrophages of the liver, uptake large amounts of free fatty acids, which contributes to their proinflammatory activation. During inflammatory activation, Kupffer cells produce proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, which are important participants in the progression of inflammation and development of NASH[68]. Thus, free fatty acids mediate the link between lipid metabolism and the innate immune system[69-71].

It is important to note that Kupffer cells, like other macrophages, have complex immunometabolic regulation (Figure 3). It has been shown that a prolonged high-fat diet increased the number of Kupffer cells with a proinflammatory M1 phenotype producing proinflammatory cytokines. Saturated fatty acids promoted M1 polarization of Kupffer cells, whereas ω-3 PUFAs polarized Kupffer cells to the M2 phenotype, which was associated with activation of the NF-κB and PPAR-γ signaling pathways, respectively[72]. The proinflammatory M1 phenotype of macrophages is characterized by enhanced glycolysis and fatty acid synthesis, whereas the anti-inflammatory M2 macrophages use fatty acid oxidation[73].

It has been suggested that polarization of M2 Kupffer cells may protect against fatty liver disease. M2 macrophages have been shown to be predominant in individuals with limited liver lesions, corresponding to little hepatocyte apoptosis compared with patients with more severe lesions[74]. Interestingly, M2-induced apoptosis of M1 macrophages is one of the mechanisms regulating the balance between M1 and M2 macrophages[74].

It has been suggested that elevated levels of free fatty acids, resulting from their excessive intake with food or by release from adipose tissue during starvation, may be the main cause of TNF release from Kupffer cells, leading to hepatocyte steatosis. Toll-like receptor 4 (TLR4) is able to detect free fatty acids on Kupffer cells to detect excess and overload of fatty acids in the liver[75]. It is known that saturated fatty acids can participate in the activation of TLR4, a receptor of the innate immune system[76-78]. This action can be associated with both direct stimulation of the receptor, confirming the evolutionary connection with the structure of LPS, which is the receptor aimed at detecting. In addition, fatty acids can be incorporated into the phospholipids of the plasma membrane and thus influence their biophysical properties and function[77,79]. The saturation and length of the alkyl chain are important.

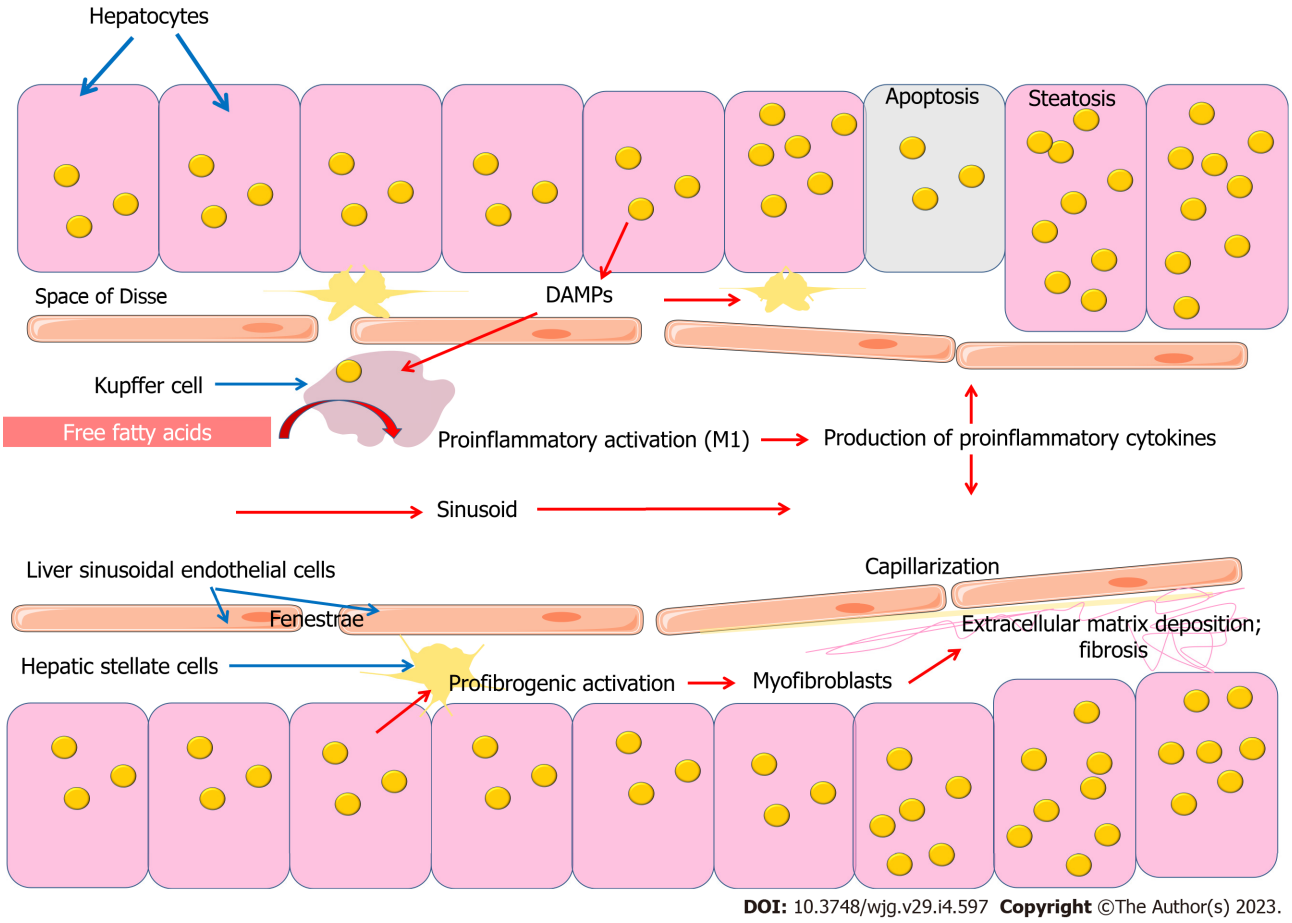

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Figure 2 Cellular mechanisms of non-alcoholic fatty liver disease pathogenesis. DAMPs: Damage associated molecular patterns.

By influencing the biophysical properties of the plasma membrane and the stability of lipid rafts in this way, the function of some membrane proteins can be regulated. It is suggested that unsaturated fatty acids contribute to a decrease in lipid ordering and the stability of lipid rafts, which may lead to anti-inflammatory effects, given the role of lipid rafts as platforms for the assembly and function of many signaling pathways. Thus, unlike saturated fatty acids, unsaturated fatty acids do not have the ability to activate TLR4. In addition, their effect on the biophysical properties of plasma membranes is opposite [77].

Unsaturated fatty acids can participate in the regulation of inflammation not only due to their biophysical properties. They are also precursors for the formation of many lipid mediators associated with inflammation. The family of lipid mediators called "specialized pro-resolving mediators" includes lipoxins, resolvins, protectins and maresins. They are formed enzymatically from ω-3 and ω-6 PUFAs such as arachidonic acid, EPA and DHA. Lipoxins are formed from arachidonic acid, E-series resolvins from EPA, and D-series resolvins, protectins and maresins from DHA[80].

Circulating maresin-1 (MaR1) levels were shown to be decreased in NAFLD patients, and a negative correlation between NAFLD and serum MaR1 concentrations was found[81]. MaR1 is mainly synthesized in M2-macrophages and plays an important anti-inflammatory role. It improves insulin sensitivity and eliminates adipose tissue inflammation[82]. In addition, MaR1 improves hepatic steatosis by inhibiting endoplasmic reticulum stress and lipogenic enzymes, and inducing autophagy *via* the AMP-activated protein kinase (AMPK) pathway[81,83,84]. Activation of Kupffer cells leads to M1 polarization and a decrease in the M2 phenotype, which corresponds to a decrease in maresin production and a decrease in their anti-inflammatory effect. Resolvin D1 (RvD1), which is an endogenous mediator produced from ω-3 DHA, reduced macrophage accumulation in adipose tissue and improved insulin sensitivity in obese and diabetic mice[85]. RvD1 shifted macrophages from an M1-to-M2-like anti-inflammatory phenotype, triggering the resolution process initiated by caloric restriction in obesity-induced steatohepatitis[86]. Protectin DX, derived from DHA, showed suppressive effects on inflammation and insulin resistance and improved hepatic steatosis by suppressing endoplasmic reticulum stress through AMPK-induced ORP150 expression[87].

On the other hand, the development of NAFLD correlates with an increase in serum eicosanoids. Moreover, profiling of plasma eicosanoids and other PUFA metabolites can differentiate NAFLD from NASH[88]. 11,12-dihydroxy-eicosatrienoic acid (11,12-diHETrE) was used as a biomarker to differ-

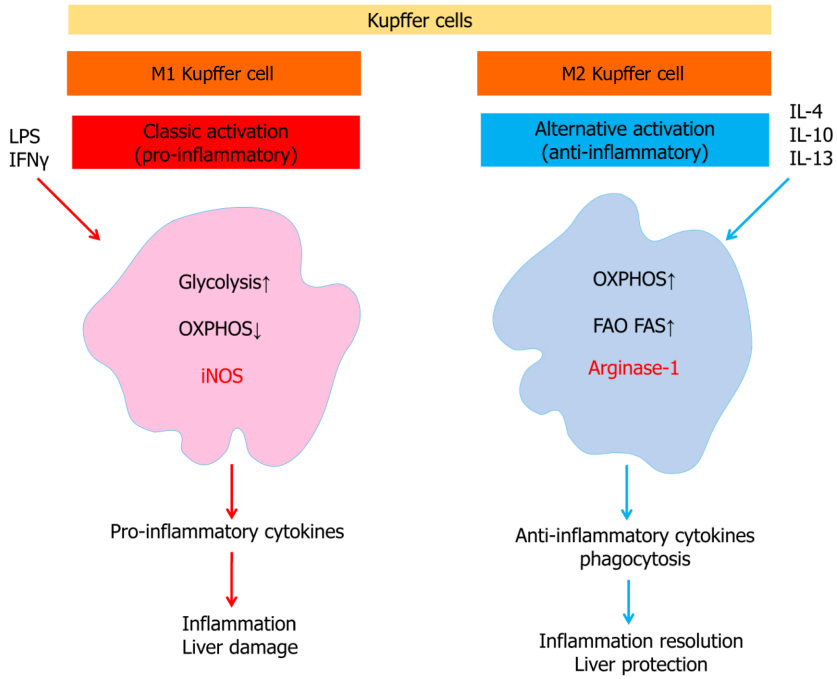

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Figure 3 Kupffer cell polarization. LPS: Lipopolysaccharide; FAO: Fatty acid oxidation; FAS: Fatty acid synthesis; IFN-γ: Interferon gamma; IL: Interleukin; iNOS: Inducible nitric oxide synthase; OXPHOS: Oxidative phosphorylation.

entiate NAFLD from NASH[88]. In another study, patients with NASH had significantly elevated levels of 9- and 13-HODE and 9- and 13-oxoODE, products of linoleic acid oxidation, compared with patients with steatosis[89]. Interestingly, patients with stage I NAFLD had lower plasma levels of 5-HETE, whereas patients with stage II steatosis had higher concentrations of 9-HODE[90].

Thus, lipid metabolites derived from fatty acids are involved in the development of NAFLD, which is an interesting topic for further research (Figure 2).

Hepatocellular accumulation of lipids can modulate the biological activity of Kupffer cells through a number of mechanisms. On the one hand, fat-saturated hepatocyte swelling changes the architecture of the sinusoidal network, reducing intrasinusoidal volume and microvascular blood flow. Disruption of microvascular blood flow also contributes to the involvement of sinusoidal endothelial cells, Kupffer cells, stellate cells and involvement of inflammatory cells and platelets[91]. Later developing fibrosing steatohepatitis with capillarization of the sinusoids, increases narrowing and distortion of the sinusoidal lumen, further limiting microvascular blood flow. In addition, leukocytes entering the narrowed sinusoids may adhere to the endothelium as a result of activation of the hepatic microvascular inflammatory response[91]. On the other hand, fat overload of hepatocytes causes lipotoxicity and the release of damage-associated molecular patterns (DAMPs), which can activate Kupffer cells and hepatic stellate cells (HSCs), promoting inflammation and fibrosis[92]. Lipid accumulation in hepatocytes has been shown to induce the release of factors that accelerate the activation and proliferation of HSCs and increase their resistance to apoptosis[93]. Conditioned medium from steatotic hepatocytes induced expression of the profibrogenic genes transforming growth factor (TGF)-beta, tissue inhibitor of metalloproteinase-1 (TIMP-1), TIMP-2 and matrix metalloproteinase-2, and expression of the NF-κB-dependent monocyte chemotactic protein-1 (MCP-1) in HSCs[93]. Thus, quiescent HSCs participate in the maintenance of liver architecture by maintaining the balance of extracellular matrix, while disruption of this balance, for example, due to metabolic disorders, leads to HSCs activation and fibrosis[94,95].

Hepatocytes exposed to apoptosis form apoptotic bodies, which are phagocytosed by HSCs and Kupffer cells, triggering a profibrogenic response due to transdifferentiation of HSCs into collagen-producing myofibroblasts[96]. Apoptotic cell uptake has been shown to stimulate Kupffer cell production of death ligands, including Fas ligand and TNF-alpha, which promotes inflammation and fibrogenesis[97].

An important pathogenetic mechanism involved in the pathogenesis of NAFLD is the role of the intestinal microbiota and a defect in the intestinal barrier caused by liver damage. Impaired gut barrier function is thought to accelerate translocation of enteric LPS, which activates proinflammatory signaling pathways and the release of related inflammatory factors in the liver[98]. Intestinal bacterial microflora and TLR4 have been shown to be involved in liver fibrogenesis[99]. *Escherichia coli* LPS can enhance liver damage in NAFLD by inducing macrophage and platelet activation through the TLR4 pathway [100]. Plasma endotoxin levels and inflammatory markers have been shown to be significantly higher in NAFLD compared with controls and to increase with the severity of hepatic steatosis[101]. Proinflam-

matory activity and immune imbalance associated with the pathophysiology of NAFLD may be related to gut dysbiosis[102]. For example, decreased Bacteroidetes and increased Firmicutes were observed in obese individuals[102]. Changes in gut microflora ratios may also increase endogenous ethanol production, which generally increases gut permeability, and contributes to translocation of endotoxins from the gut lumen into the portal bloodstream[102,103].

Another immunometabolic link between the gut microbiota and NAFLD, related to short-chain fatty acids (SCFAs), should also be noted[104,105]. SCFAs are formed by the gut microbiota during the fermentation of non-digestible fibers such as resistant starch, cellulose, and pectin[106]. SCFAs are used by colonic mucosal epithelial cells as an energy substrate, are involved in the regulation of a number of processes in the intestinal wall or enter the portal bloodstream, and may be involved in the formation of immunometabolic connections with other organs[107].

A growing body of evidence strengthens the understanding of the importance of SCFAs in inflammation. SCFAs act *via* receptors associated with the G-protein GPR43 and GPR41, also known as free fatty acid receptor (FFA)2 and FFA3, respectively[108-111]. In addition, SCFAs realize their action through inhibition of histone deacetylase (HDAC)[112,113].

Butyrate is well known for its anti-inflammatory properties and is of great clinical interest[107,114,115]. Through HDAC3 inhibition, butyrate can induce a metabolic switch of macrophages toward an anti-inflammatory M2 phenotype[112,113].

SCFAs are also known to affect the differentiation, recruitment and activation of neutrophils, DCs, macrophages and monocytes as well as T cells[116,117]. Butyrate is involved in the regulation of DC differentiation derived from human monocytes, keeping DCs in the immature stage[118].

In addition to their involvement in inflammation, SCFAs regulate lipid metabolism in the liver. Butyrate levels have been shown to decrease in NAFLD patients and mice with decreased estrogen levels, with butyrate administration attenuating liver steatosis[119]. Studies in rats fed a high-fat diet (HFD) have shown that butyrate increases β-oxidation of fatty acids, inhibits lipid synthesis and suppresses nuclear factor-kappa B and inflammation[120,121]. The addition of sodium butyrate protects mice from developing NASH. It is important to note that the metabolic role of SCFAs in liver function is rather complex[122]. In addition to attenuating hepatic steatosis, acetate, another SCFA derived from the microbiota, may conversely promote hepatic lipogenesis after excessive fructose intake[123,124].

A growing body of evidence strengthens the understanding that lipoproteins are part of an important transport mechanism that is utilized by the innate immune system. The mechanism of LPS elimination involves LPS disaggregation and binding to circulating lipoproteins, uptake of lipoprotein-associated LPS by the liver, and excretion of LPS with the bile[125,126]. This pathway, known as reverse LPS transport, involves lipoproteins as the main carriers of LPS in the plasma and includes the proteins LBP, BPI, phospholipid-transfer protein (PLTP), and cholesteryl ester transfer protein (CETP), which belong to the lipid transfer/LPS binding gene family (LT/LBP) and play different roles in LPS metabolism [126]. In addition, reverse cholesterol transport is at the beginning of the cross-talk between cholesterol metabolism and the innate immune system[126]. ABCA1, a key participant in reverse cholesterol transport also contributes to the efflux of LPS from macrophages[127]. HDL and other plasma lipoproteins have been shown to contribute to the release of LPS from the cell surface of monocytes[128].

Lipid transfer proteins (lecithin-cholesterol acyltransferase (LCAT), CETP, and PLTP) as well as hepatic and endothelial lipases remodel HDL in the bloodstream. CETP is part of a family of proteins including LPS-binding protein (LBP) and bactericidal permeability increasing protein (BPI) and may participate in the transport of LPS between lipoproteins for further utilization in the liver. CETP transports cholesterol esters from HDL to apoB-containing LDL and very low density lipoproteins (VLDLs).

Kupffer cells take up most of the LPS and can inactivate it by deacylation with acyloxyacyl hydrolase. Kupffer cells express high levels of class A scavenger receptors (SR-A), which bind oxidized low-density lipoproteins (LDL) and are also involved in LPS uptake[64,129]. SR-A expression is increased by oxidized LDL[130,131]. Importantly, in the liver, SR-A is also important for cell adhesion, suggesting a role for SR-A in the recruitment and retention of cells in various organs or in sites of pathological conditions, such as foci of inflammation or areas of atherosclerotic lesions[64]. In addition to Kupffer cells, SR-A types I and II are expressed in the liver on endothelial cells, which are less able to bind LPS [132].

Interestingly, plasma CETP predominantly originates from Kupffer cells, and plasma CETP levels predict the content of Kupffer cells in the liver in humans[133]. In addition, activation of Kupffer cells by LPS strongly decreases CETP expression[134]. LPS has been shown to activate resting Kupffer cells, resulting in decreased hepatic CETP expression and decreased CETP in plasma and increased HDL cholesterol levels[135]. Importantly, CETP inhibition improves HDL function but leads to liver obesity and insulin resistance in CETP-expressing transgenic mice on a HFD[136]. Information obtained in recent years has improved the understanding of the role of CETP in inflammation. Experimental evidence suggests that CETP in macrophages as well as in the liver prevents LPS interaction with TLR4, thereby reducing the inflammatory response[137]. Compared with wild-type mice, CETP mice showed a higher survival rate after polymicrobial sepsis. CETP mice had lower plasma IL-6 concentrations and decreased levels of hepatic TLR4 and acyloxyacyl hydrolase protein[137]. Species-specific differences in CETP expression should be noted[138]. In mice and rats, in contrast to humans, as well as primates,

rabbits, and hamsters, CETP is absent in plasma. Consequently, wild-type mice, have naturally low LDL and high HDL levels, in which up to 90% of cholesterol is transported and have low susceptibility to developing atherosclerosis. Transgenic mice expressing human CETP have increased reverse cholesterol transport, which is associated with increased clearance of apoB lipoproteins in the liver. They also show increased postprandial triglyceridemia, increased liver uptake of LPS, and increased survival in endotoxemia[139,140]. Transgenic expression of CETP in mice also reduces liver fat accumulation and improves insulin sensitivity in diet-induced obesity[141,142]. CETP has been shown to reduce liver TAG content in female mice through enhanced β-oxidation and to promote the synthesis and assembly of VLDL[142]. CETP inhibition in transgenic CETP-expressing mice disrupted TAG metabolic pathways, leading to liver TG accumulation and insulin resistance in diet-induced obese mice[136]. In addition, CETP inhibition by anacetrapib increased systemic and hepatic inflammation to a greater extent in obese mice[136].

Despite its weaker ability to bind LPS compared to LBP or BPI, CETP is associated with resistance to sepsis. Experiments with human CETP transgenic mice showed lower mortality after LPS administration compared to wild-type mice. The pathway involving CETP is of interest because it represents a cross-talk mechanism of reverse cholesterol transport and the innate immune system, in which LPS and cholesterol share common transport and utilization pathways[140].

Neutrophils, other important participants in the innate immune system, are also involved in the pathogenesis of NAFLD[143]. Given that inflammation is a key event that contributes to the progression of fatty liver dystrophy to NAFLD, these patients show significant neutrophil infiltration into the liver, often accompanied by increased expression of chemokines that promote neutrophil chemotaxis[144].

Neutrophils exhibit cross-links with HSCs. On the one hand, neutrophils activate HSCs through the production of ROS[145-147]. On the other hand, activated HSCs have been shown to support neutrophil survival by producing granulocyte-macrophage colony-stimulating factor and IL-15. This may serve as a positive direct loop contributor to liver damage and fibrosis under a HFD[147].

Interestingly, it has been shown that neutrophils in blood in patients with NASH had increased expression of receptors reflecting the preparation of neutrophils to migrate into tissue. In addition to preparation for migration, blood neutrophils in NASH were also functionally activated[148]. They were characterized by increased IL-8 production and had more than double the spontaneous oxidative burst. In analyzing these data, it was noted that neutrophils can not only move from the vascular lumen into extravascular tissues but can also move back into the bloodstream, through a process known as reverse transendothelial migration. Reverse transendothelial migration is of interest due to its possible interaction with the immune system[149]. However, its possible role in NAFLD has yet to be studied.

Thus, neutrophils play an important role in the development of inflammation and liver fibrosis[150]. On the other hand, neutrophils contribute to the spontaneous resolution of inflammation and liver fibrosis. Acting *via* miR-223, neutrophils act as resolving effector cells that induce the transition of proinflammatory macrophages to a restorative phenotype by suppressing NLRP3 inflammasome expression[151]. Another study in a diet-induced NASH mouse model also showed a phase-dependent contrasting role of neutrophils as triggers and pro-resolutive mediators of liver injury and fibrosis[150]. In addition to these findings, miR-223 was shown to be elevated in hepatocytes from HFD-treated mice and patients with NASH, which may be due to the fact that miR-223 can be transferred from neutrophils *via* the exosome. Moreover, miR-223 in hepatocytes acts as an anti-inflammatory molecule, directly affecting several inflammatory genes[152].

Thus, neutrophils play a complex multifaceted role in the pathogenesis of NAFLD, which is a promising topic for further research.

Liver DCs are a heterogeneous population of hepatic sinusoidal antigen-presenting cells[153,154]. DCs exist in mature or immature states and undergo maturation when exposed to immune or inflammatory signals such as microbial products and proinflammatory cytokines. DCs are involved in maintaining immune homeostasis and liver tolerance by promoting CD8+ T-cell elimination, as well as secreting anti-inflammatory cytokines that maintain the quiescent HSC state and promote TLR4 refractoriness to LPS. In addition, DCs regulate the number and activity of cells involved in the development of fibrosis and may play a role in the regression of liver fibrosis[155]. Dendritic cells can contribute to liver fibrosis regression by activating metalloproteinases and contribute to the homeostasis of NK cells, which are mainly antifibrogenic[154].

Natural killer cells are a heterogeneous multifunctional population of lymphoid cells located inside the sinusoidal space, where they can attach to endothelium and Kupffer cells[156]. A key factor determining the activity of these cells in NASH is their metabolic reprogramming.

Liver NK cells are part of the innate immune system and may play an important role in NAFLD. However, the regulation and function of NK cells in NAFLD remains controversial due to their different involvement at different stages of the disease. On the one hand, NK cells are active and may be useful in the early stages of fibrosis, when they contribute to TRAIL-mediated HSC death. On the other hand, NK cell involvement becomes detrimental when they lose their antitumor capacity, which may contribute to disease progression in later stages[156]. Indeed, metabolic reprogramming of NK cells in obesity limits the antitumor response, which is known as "metabolic paralysis"[157]. Overload of NK cells with lipids absorbed from the environment in obesity leads to metabolic defects that cause inhibition of the cytotoxic mechanism, resulting in loss of antitumor functions[157]. Overall, the available data suggest a

possible therapeutic potential for the regulation of NK cell function, which is a promising topic for further research.

ROLE OF RECEPTORS IN THE INNATE IMMUNE SYSTEM

The innate immune system relies on a large number of pattern recognition receptors (PRRs) to recognize both DAMPs and pathogen-associated molecular patterns. Toll-like receptors (TLRs) are the most well characterized representatives of PRRs. They are expressed in a variety of liver cells, including Kupffer cells, HSCs, hepatocytes, sinusoidal endothelial cells, and biliary epithelial cells[158-160]. A growing body of evidence reinforces the importance of TLRs in the pathogenesis of NAFLD[161]. TLR4 is of particular interest in connection with liver inflammation and fibrogenesis[158,162,163]. TLR4 is a receptor that detects the LPS of Gram-negative bacteria and is widely known for its role in various diseases.

TLR4 is expressed on all types of liver cells, including Kupffer cells, HSCs, and hepatocytes. Under normal conditions, hepatic cells express minimal TLRs, indicating a high tolerance of the liver to TLR ligands[164]. At the same time, receptor expression in the liver is associated with inflammation and fibrosis[164]. TLR4 plays a central role in Kupffer cell activation by responding to LPS. LPS is considered a potent inducer of hepatic inflammation. It promotes the production of TNF-α in Kupffer cells, which is a mediator of inflammation in the pathogenesis of NAFLD[165]. In addition, LPS can activate HSCs, and Kupffer cells can enhance this process by producing TGF-β and making HSCs more sensitive to TGF-β[164]. Despite the fact that Kupffer cells are the main targets for LPS in the liver, it is HSCs that contribute to TLR4-dependent fibrosis[99]. In addition, modulation of TGF-β signaling along the TLR4-MyD88-NF-κB axis provides a link between proinflammatory and profibrogenic signals[99]. Numerous data support the involvement of HSCs as central mediators of hepatic fibrosis. Activation of TLR4 in quiescent HSCs enhances chemokine secretion and induces Kupffer cell chemotaxis and inhibits the TGF-β pseudoreceptor Bambi, which increases HSCs sensitivity to signals induced by TGF-β and enables unrestricted activation by Kupffer cells[99]. A significantly reduced expression of the Bambi gene in HSCs was seen when incubated with the TLR4 LPS ligand[166].

It was found that a diet high in cholesterol leads to the accumulation of free cholesterol in HSCs, which promotes TLR4 signaling by increasing TLR4 levels in the membrane and can suppress Bambi gene expression. As a consequence, TGF-β signaling in HSCs was enhanced, leading to HSCs activation and progression of liver fibrosis[166].

ENDOTHELIAL CELL INVOLVEMENT IN THE IMMUNE SYSTEM IN THE LIVER

Endothelial cells, which form the inner membrane of blood vessels, play an important role in the functioning of the barrier between blood and tissues. Endothelium is characterized by heterogeneity and plasticity due to phenotypic specialization of different tissue types. This endothelial specialization can provide dense connections necessary for functioning of histo-tissue barriers, or on the contrary can promote infiltration and extravasation of molecules and particles circulating in the bloodstream due to fenestrated endothelium in the liver and kidneys[167]. Given that the liver is a highly vascularized organ (accounting for 20% of cardiac output), hepatic sinusoidal endothelial cells constitute a significant proportion of the total number of liver cells[168]. Liver sinusoidal endothelial cells (LSECs) have a unique morphological phenotype characterized by a combination of numerous fenestrae and lack of a basement membrane, which provides open access for dissolved substances between the sinusoidal blood and the Disse space (Figure 2). LSECs are involved in regulation of the liver microenvironment and act as the liver's first protective barrier. An important functional phenotypic feature of hepatic sinusoidal endothelial cells is their high endocytic capacity[169]. These cells are capable of absorbing and removing soluble macromolecules from the portal venous blood in addition to Kupffer cells located on the lumen side of the endothelium[168].

Disruption of the LSECs phenotype is a critical step in the liver fibrosis process (Figure 2). Capillarization, in which there is a lack of fenestration of hepatic sinusoidal endothelial cells and formation of an organized basal membrane, precedes fibrosis and contributes to HSC activation[169]. Vascular endothelial growth factor (VEGF) produced by hepatocytes and HSCs has been shown to be a key regulator of the LSEC phenotype[169-173]. The maintenance of the fenestrated LSEC phenotype is provided by the action of VEGF through a nitric oxide (NO)-dependent and NO-independent pathway [169-171]. In this case, VEGF, which is produced by hepatocytes or stellate cells, promotes NO formation from LSECs *via* endothelial nitric oxide synthase (eNOS)[171].

A growing body of evidence supports the important role of the endothelium in vascular biology. Endothelial cells can detect changes in blood flow and are involved in the regulation of hemodynamics and inflammation through the production of several bioactive substances. Endothelial production of NO is the best known way to regulate vascular hemodynamics. Nitric oxide is an important signaling molecule that is at the crossroads between the regulation of vascular hemodynamics and innate

immunity[174]. Importantly, NO demonstrates active involvement in the regulation of inflammation in the vascular wall, which is important in the development of atherosclerosis. Endothelial NO actively regulates the innate immune response involved in atherogenesis by regulating macrophage and lymphocyte uptake and vessel wall migration *via* adhesion molecules[174].

Nitric oxide synthesis in the endothelium is carried out by a specific constitutive eNOS isoform. Mechanical stimulation of endothelial cells by blood flow triggers a complex chain of events involving numerous cellular mechanosensors and enzymes, leading to activation of eNOS[175]. eNOS is expressed in LSECs and produces small amounts of NO, which maintain intrahepatic sinusoidal vascular tone and hemodynamics in the liver. Another isoform of nitric oxide synthase, inducible NOS (iNOS) is expressed in various liver cells, including LSECs, hepatocytes, Kupffer cells, HSCs and other immune cells[176-178]. LPS induces iNOS expression and NO production and increases caveolin-1 and decreases eNOS phosphorylation[179].

It should be noted that while the NO produced by eNOS has a hepatoprotective effect by inhibiting inflammatory activation of Kupffer cells, the NO produced by iNOS, in contrast, promotes NAFLD [180]. iNOS produces significantly more NO than eNOS, which can have negative effects. This is due to the cytotoxicity of NO in high concentrations. In particular, peroxynitrite (ONOO-) can damage a wide range of cellular molecules[181]. Interestingly, peroxynitrite can affect cyclooxygenase (COX)-1 and COX-2 activity depending on the concentration[182,183]. It has been suggested that NO can interact directly with COX, for example *via* S-nitrosylation, causing an increase in its enzymatic activity[184, 185]. Thus, NO production has closely overlapping connections with innate immunity. These and other data suggested a role for COX enzymes as important endogenous receptor targets for NO functions [186]. COX-2-mediated inflammation is important for insulin resistance associated with obesity and fatty liver dystrophy. Daily aspirin intake was associated with less severe histologic signs of NAFLD and NASH and a reduced risk of fibrosis progression over time[187].

Importantly, eNOS activity is decreased in pathological conditions, whereas iNOS activity is increased. Decreased NO production in LSECs causes endothelial cell capillarization and HSCs activation. This leads to deposition of extracellular matrix, proliferation of HSCs, increased intrahepatic resistance and impaired sinusoidal blood flow[180].

Thus, the function of NO is related to the maintenance of liver cell function. NO derived from eNOS protects against liver disease, whereas NO derived from iNOS has a proinflammatory effect[180]. When mice were fed a HFD, a decrease in liver NO was shown to precede the onset of liver inflammation through the NF-κB pathway as well as impaired insulin signaling at the IRS-1 and phospho-Akt levels. Thus, an important physiological role of endothelial NO has been shown to limit obesity-associated inflammation and impaired insulin signaling in hepatocytes and Kupffer cells *via* the NO/cGMP-dependent protein kinase (PKG)/ vasodilator-stimulated phosphoprotein (VASP) pathway as part of a cross-talk mechanism with metabolic disturbances associated with obesity[168].

LSECs exhibit a proinflammatory phenotype during the progression of NAFLD to NASH. It is characterized by surface overexpression of adhesion molecules such as ICAM-1, VCAM-1, and VAP-1 (AOC3) and production of proinflammatory molecules such as TNF-α, IL-6, IL-1, and MCP1 (CCL2)[188,189]. Interestingly, LSECs and HSCs are involved in maintaining each other's differential phenotype. On the one hand, VEGF-A production by either HSCs or hepatocytes supports LSECs differentiation[170]; on the other hand, fenestrated LSECs prevent HSCs activation and promote the conversion of activated HSCs to a dormant state. However, LSECs lose this effect when they are undifferentiated or have a capillarized phenotype[171,190].

Thus, LSECs play an important role in liver immunology and the development of NAFLD. In contrast to hepatocytes, free fatty acids such as palmitic acid and oleic acid inhibit LPS-induced production of proinflammatory chemokines in LSECs and inhibit inflammatory cell recruitment. These findings suggest a potentially protective role for LSECs in the liver with excess free fatty acids, as in NAFLD [191].

A growing body of evidence suggests that the role of lipid metabolism in endothelial cell function is not only as a structural or energetic substrate, but also as a participant in cell mechanobiology. In doing so, lipids are at the intersection of chemo- and mechanobiological signaling pathways.

CONCLUSION

NAFLD is a widespread disease whose clinical and pathophysiological links are only beginning to be understood. TAG accumulation in hepatocytes in NAFLD results from a complex chain of events and is complicated in nature, involving many exogenous and endogenous factors. Obesity and impaired lipid metabolism are considered to be the key links in the development of NAFLD. Moreover, impaired fatty acid metabolism is one of the central events in the pathogenesis of NAFLD due to their involvement not only as an energy substrate or their structural function in cells, but also due to their connection with the innate immune system. Lipid metabolism has multiple cross-links with the innate immune system, and these links are important in the pathogenesis of NAFLD.

Analysis of the data allows us to emphasize the need for a better study of the multifaceted role of lipid metabolism and its disorders as a link in the complex chain of processes underlying the development of NAFLD.

The pathogenesis of NAFLD is an important target for further research, among which immunometabolic cross-linkages can be considered as one of the promising directions. Immunometabolic regulation of cells and intercellular connections at different stages of liver disease development can be a significant target for therapeutic intervention. In addition, the immune and metabolic axes that link the liver to other organs are also of research and clinical interest. There is a growing understanding that the gut microbiota is an important participant in immune and metabolic processes not only in the gut, but also in other organs. There is also interest in information on the cross-linkages of lipid-transport function and innate immunity, which have evolutionarily conservative roots and link a number of diseases that mutually influence their natural history.

In summary, NAFLD is a complex multifaceted disease whose keys are still unknown to clinicians and researchers, but a better understanding of metabolic and immune cross-linkages will improve patient diagnosis and treatment approaches.

FOOTNOTES

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REVIEW

Iron as a therapeutic target in chronic liver disease

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Abstract

It was clearly realized more than 50 years ago that iron deposition in the liver may be a critical factor in the development and progression of liver disease. The recent clarification of ferroptosis as a specific form of regulated hepatocyte death different from apoptosis and the description of ferritinophagy as a specific variation of autophagy prompted detailed investigations on the association of iron and the liver. In this review, we will present a brief discussion of iron absorption and handling by the liver with emphasis on the role of liver macrophages and the significance of the iron regulators hepcidin, transferrin, and ferritin in iron homeostasis. The regulation of ferroptosis by endogenous and exogenous modulators will be examined. Furthermore, the involvement of iron and ferroptosis in various liver diseases including alcoholic and non-alcoholic liver disease, chronic hepatitis B and C, liver fibrosis, and hepatocellular carcinoma (HCC) will be analyzed. Finally, experimental and clinical results following interventions to reduce iron deposition and the promising manipulation of ferroptosis will be presented. Most liver diseases will be benefited by ferroptosis inhibition using exogenous inhibitors with the notable exception of HCC, where induction of ferroptosis is the desired effect. Current evidence mostly stems from *in vitro* and *in vivo* experimental studies and the need for well-designed future clinical trials is warranted.

Key Words: Iron overload; Liver disease; Ferroptosis; Ferritinophagy; Ferroptosis modulators

Core Tip: Iron overload may damage the liver in a variety of liver diseases such as cirrhosis and hepatocellular carcinoma affecting patient survival. In this review, we present the evidence, both experimental and clinical, of the detrimental effects of iron deposition in hepatocytes and other liver sinusoidal cells. Moreover, we examine the mechanism and implications of the recently described ferroptosis in the evolution of liver disease. Ferroptosis is a form of regulated hepatocyte death caused by excess iron and lipid peroxidation. Inhibition or induction of ferroptosis may profoundly improve the course of many liver diseases as demonstrated by a large number of experimental studies as well as a small number of clinical trials.

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INTRODUCTION

The major suppliers of plasma iron are duodenal enterocytes and iron-recycling macrophages[1-3]. Duodenal cytochrome B reductase reduces inorganic trivalent iron reaching the duodenum to form divalent iron (Fe^{2+}), and surface divalent metal transporter 1 (DMT1) imports Fe^{2+} into the cytoplasm. The gene *SLC11A2* encoding DMT1 is activated in cases of iron deficiency or hypoxia as it interacts with the hypoxia-inducible factors (HIF1α and HIF2α) overexpressed in these situations[4-7]. The cytoplasmic iron sensor iron-responsive element (IRE) and iron regulatory proteins (IRP1 and IRP2) also participate in iron absorption control as they stabilize the *SLC11A2* transcript in iron deficiency or dissociate and degrade in iron overload[8]. Then, the cytoplasmic divalent iron is bound to ferroportin, the only known iron exporter protein, and exported to the portal vein blood. Transportation is mediated by the chaperone protein poly (rC)-binding protein 2 encoded by the *SLC40A* gene[9]. The main regulator of ferroportin is hepcidin[10], but the IRP/IRE proteins and microRNAs are also involved[11]. Once in the portal vein, the divalent iron is oxidized back to trivalent by the ferroxidases hephestin and ceruloplasmin and then carried in different cells bound to transferrin. Peripheral cells import iron by the internalization of transferrin after it binds to its receptor TFR1[12] and is sorted into endosomes where iron is removed in the acidic environment, reduced again to $Fe2^{+}$ by the ferrireductase STEAP3, and released into the cytosol by DMT1[1,3]. Iron is then either exported by ferroportin or stored in ferritin or in the labile iron pool (LIP). On the other hand, heme oxygenases (Hos) localized mainly in iron-recycling macrophages of liver and spleen, degrade heme to recover Fe^{2+}[2,13].

The regulation of hepcidin is critical in iron metabolism as binding of hepcidin to ferroportin in hepatocytes, macrophages, or enterocytes leads to internalization and degradation of ferroportin, thus limiting iron export to the blood[2,3,10]. A decrease in hepcidin when iron is needed leads to enhancement of ferroportin expression and increased iron absorption from the duodenum. In iron overload, ferroportin is downregulated and iron absorption is decreased[14]. In addition to iron deficiency, inflammatory molecules like interleukin (IL)-6 also upregulate hepcidin expression[15]. The *HAMP* gene encodes hepcidin, and its promoter is activated by the complex of bone morphogenic proteins (BMP2, BMP4, BMP6) and their receptor. This complex phosphorylates the SMAD pathway, which in turn activates *HAMP* expression[16,17]. Hemojuvelin (HJV) is a necessary co-factor for BMP-BMP receptor complex function[18]. BMP6 is mainly expressed in liver sinusoidal cells and induces hepcidin upregulation *via* paracrine signaling during iron overload[19-21].

The second receptor of transferrin (TFR2), a low-affinity receptor found in hepatocytes and erythroid precursors, is also an important inducer of hepcidin through the BMP/SMAD pathway[22-24] after forming a complex with HFE (the protein involved in hereditary hemochromatosis)[25]. Anomalies of either of the genes encoding these proteins will lead to hepcidin downregulation[26-28].

HEPCIDIN INHIBITORS

In contrast to iron overload, hypoxia, anemia, and erythropoiesis reduce hepcidin expression[29,30]. The main inhibitor of hepcidin expression is erythroferrone (ERFE)[31], which is produced by erythroid cells in response to erythropoietic stimuli. ERFE downregulates hepcidin, interfering with the BMP/SMAD pathway in hepatocytes[32-34]. Three other hepcidin inhibitors have been described. PIEZO1 and the immunophilin FKBP reduce *HAMP* expression by inhibiting the BMP/SMAD pathway[35,36]. The third hepcidin inhibitor is the ferritinophagy axis operating in both the enterocyte and the macrophage. Ferritinophagy is a specialized form of autophagy resulting in the lysosomal breakdown of ferritin and subsequent iron release to increase the LIP. It is controlled by the nuclear receptor coactivator 4

(NCOA4)[37,38] during transport of absorbed iron to ferritin. In increased iron demand, NCOA4 functions as a cargo receptor for lysosomal degradation of ferritin. Excess iron leads to lipid peroxidation-mediated ferroptosis[38]. NCOA4 is similarly involved in macrophage ferritinophagy and iron release for erythropoiesis[39].

Iron ions are dangerous to cells. In iron overload, redox-active iron increases and oxidative stress is induced through the formation of reactive oxygen species (ROS). Non-transferrin bound iron is mainly responsible for the redox-active iron when the capacity of iron binding proteins is not able to accommodate for the increased iron load. An additional dangerous form is the transit iron pool, which comprises iron that is not bound to ferritin or other chelating proteins. This iron may also induce the formation of ROS[40]. Iron is a double-edged sword[41], which even under normal conditions may cause pathological damage. Iron induces hydroxyl radical production through the Fenton reaction[42]. The Fenton-Haber-Weiss reaction is caused by the free donation and acceptance of electrons during the transition between Fe^{2+} and Fe^{3+} states. Iron-catalyzed generation of hydroxide ions and hydroperoxyl and hydroxyl radicals is the result of this exchange. Under normal conditions, free-radicals are quenched by cellular antioxidant mechanisms[43]. However, when overproduced, these free radicals promote the formation of other ROS such as thiyl and peroxyl radicals and a vicious circle is initiated leading to oxidation of lipids, proteins and nucleic acids[44]. Thus, in iron-loaded animals the products of lipid peroxidation such as malondialdehyde (MDA), isoprostanes, and 4-hydroxynonenal (4-HNE) can be detected in the liver[45]. MDA and 4-HNE form mutagenic adducts, reacting with amino groups and DNA bases[46,47] that target the p53 tumor suppressor gene initiating apoptotic resistance to the cells[48]. Levels of 4-HNE correlate well with hepatic iron levels[49]. Iron metabolism was recently reviewed in detail[50-53].

FERROPTOSIS

The most important mechanism of iron-induced liver damage is the recently described ferroptosis, a name derived from the Greek word "ptosis," meaning a fall, and the Latin "ferrum" or iron[54]. It is an iron-dependent regulated cell death characterized by iron accumulation, lipid peroxidation, and the production of ROS that depends on the activity of NADPH oxidases[55,56]. The mitochondrial respiratory chain initiates lipid peroxidation by lipoxygenase (LOX) or cytochrome P450 reductase. The enzyme glutathione peroxidase 4 (GPX4), the antioxidant glutathione (GSH), the coenzyme Q10 (CoQ10), and the tetrahydrobiopterin (BH4) system are the defense mechanisms of the cell. They are further regulated by the nuclear factor erythroid 2-related factor (Nrf2)[57-59]. The process is controlled by multiple genes associated with iron uptake[60,61], lipotoxicity[62,63], and antioxidation responses [64,65].

Ferroptosis is regulated by several metabolic events such as lipogenesis and ferritinophagy. The mitochondrial tricarboxylic acid cycle fueled by glutaminolysis may promote ferroptosis induction. Phospholipid peroxidation is the critical event in ferroptosis. Production of ROS, iron, and phospholipids containing polyunsaturated fatty acids (PUFA-PLs) are the necessary requirements. The executioner of ferroptosis is phospholipid hydroperoxide (PLOOH) synthesized from its precursor, PUFA[66].

Both non-enzymatic/exogenous and enzymatic/endogenous pathways are implicated in lipid peroxidation. For the latter, LOXs and/or cytochrome P450 oxidoreductase mediate the induction of lipid peroxidation by the dioxygenation of lipids. Exogenous transporter mediated signaling pathways include the E cadherin-NF2-Hippo-YAP pathway, the glucose-regulated AMPK signaling pathway, and the p53 tumor suppressor pathway[67].

Mechanisms inhibiting ferroptosis are provided by three main biological pathways (Figure 1)[68,69]. The first is the GSH/GPX4 pathway, implicating the system Xc-, which is a membrane cystine/glutamate exchanger that imports cystine and exports glutamate. A critical mediator in this system is the cystine/glutamate antiporter SLC7A11, and GPX4 is the major protective system against lipid peroxidation[70]. In addition, ferroptosis suppressor protein 1 acts mainly on the plasma membrane, and dihydroorotate dehydrogenase is an important defense molecule in mitochondria[71-75]. The second comprises iron metabolism pathways, particularly the p62-Kelch-like ECH-associated protein 1 (Keap1)-Nrf2 regulatory pathway[54]. Inhibition of ferritinophagy increases mitochondrial ferritin and protects from ferroptosis as evidenced in hypoxic macrophages. This is regulated by a hypoxia-induced decrease of *NCOA4* transcription, in combination with a microRNA 6862-5p-dependent degradation of NCOA4 mRNA[76]. Nrf2 is a transcription factor that protects cells against oxidative and toxic damage and plays a significant role in regulating ferroptosis[77-79]. In hepatocellular carcinoma (HCC) and other tumors, activation of the p62-Keap1-Nrf2 pathway leads to reduced Nrf2 degradation, the protection of tumor cells against ferroptosis, and resistance to anticancer drugs[80]. The third includes lipid metabolism pathways implicating p53 and various enzymes[54,66]. p53 is a tumor suppressor transcription factor that may prevent cancer by controlling the cell cycle, cellular senescence, and apoptosis. Ferroptosis is one of its antitumor mechanisms; p53 increases cell sensitivity to ferroptosis through repression of *SLC7A11*. The ferroptosis inhibitor fer-1 reverses this effect and induces *SLC7A11*

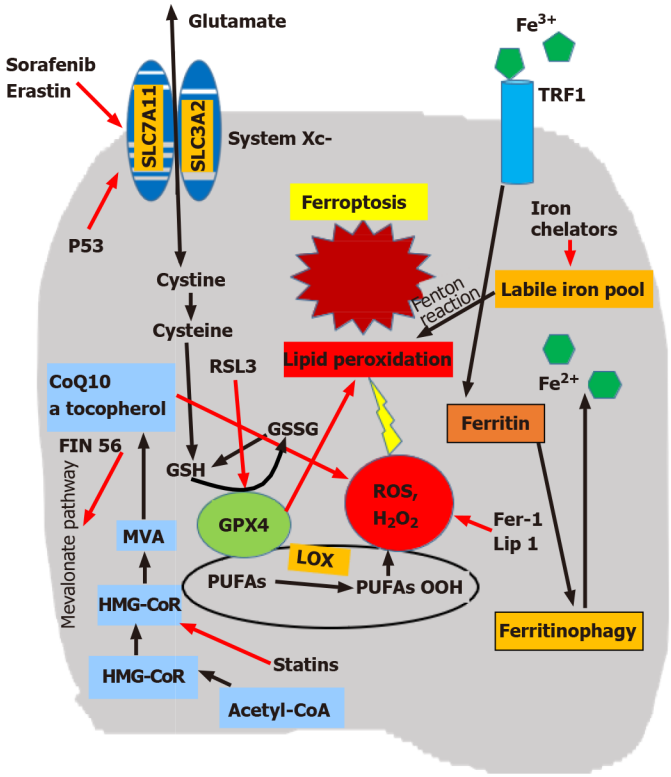

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Figure 1 Simplified pathways of ferroptosis and site of action of the main ferroptosis modulators. Ferroptosis is mediated through excess intracellular iron in the labile iron pool (LIP). Ferritinophagy is a major source of LIP, leading to the production of reactive oxygen species (ROS) by mitochondria and lipid peroxidation. Deranged function of the system Xc- causes reduction of glutathione (GSH) and glutathione peroxidase 4 (GPX4) and an increased activity of lipoxygenases (LOXs), augmenting lipid peroxidation. Ferroptosis modulators act by interfering with components of this complicated pathway. Red arrows indicate inhibition. CoQ10: Coenzyme Q10; GSSG: Glutathione disulfide; MVA: Modified vaccinia virus Ankara; PUFAs: Polyunsaturated fatty acids; SLC3A2: Solute carrier family 3 member 2; SLC7A11: Solute carrier family 7 member 11; TRF1: Transferrin receptor 1.

overexpression[62,81-83]. Additional biological factors inhibiting ferroptosis were also recently identified: (1) GTP Cyclohydrolase-1 (the rate-limiting enzyme for biosynthesis of tetrahydrobiopterin (BH4), which counteracts ferroptosis[84]); (2) Transferrin and its cell surface TFR1 receptor[12]; and (3) CDGSH (iron sulfur domain 1, which negatively regulates ferroptosis protecting against lipid peroxidation in mitochondria)[85].

EXOGENOUS FERROPTOSIS MODULATORS

Exogenous ferroptosis modulators discussed here are summarized in Table 1. Ferroptosis inhibitors are divided into two major groups: (1) Class I inhibitors, such as Deferoxamine (DFO) mesylate[86], which suppresses iron accumulation; and (2) Class II inhibitors, including ferrostatin-1, liproxstatin-1 and vitamin E, which react with chain free radicals and can inhibit lipid peroxidation[87-91]. The activity of drugs in the first generation of ferrostatin class specifically reduce the accumulation of lipid ROS. The second generation (SRS 11-92) and the third generation (SRS 16-86) ferrostatin drugs function through conferring increased metabolic stability[90].

The recently described inhibitor dynasore has characteristics of both classes, and prevents both iron accumulation and lipid peroxidation[92]. Other inhibitors of ferroptosis have been identified. For example, the cholesterol-reducing drug probucol was found to suppress ferroptosis[93]. The RIPK1 inhibitor necrostatin-1, which suppresses necroptosis, also has the additional effect of suppressing ferroptosis. Selenium administration has also been seen to suppress ferroptosis during stroke[94], and the nitroxide XJB-5-131 targets mitochondria and suppresses both apoptosis and ferroptosis[95]. However, it should be noted that these inhibitors have not been tested in the liver.

Interestingly, a recent experimental finding showed that the mechanism of action of bicyclol, a common hepatoprotectant in China, is *via* the prevention of ferroptosis. Furthermore, bicyclol attenuates cellular damage and lipid peroxidation induced by erastin. Additionally, Nrf2 inhibition and the subsequent reduction of GPX4 levels impedes the effects of bicyclol[96]. Finally, the anti-diabetic drug rosiglitazone inhibits ferroptosis and reduces hepatocyte death, acting as an ACSL4 inhibitor[97,98].

Table 1 Exogenous modulators of ferroptosis

Inducers	Mechanisms	Compounds
Class 1	Inhibition of system Xc-	Erastin, sorafenib, sulfasalazin
	Prevention of cystine import	Glutamate
Class 2	Inhibition of GPX4	RLS3, DPIs (DPI7, DPI10)
Class 3	Degradation of GPX4	FIN56
	Depletion of CoQ10	
Class 4	Initiation of lipid peroxidation	FINO2, PUFAs
	Indirect reduction of GPX4 activity	
Inhibitors		
Class 1	Suppression of iron accumulation	Deferoxamine
Class 2	Inhibition of lipid peroxidation	Ferrostatin-1, liproxstatin-1, vitamin E
Unclassified		Dynasore, probucol, selenium, nitroxide XJB-5-131
		Bicyclol, rosiglitazone

CoQ10: Coenzyme Q10; FIN: Ferroptosis inducing compounds; GPX4: Glutathione peroxidase 4; PUFAs: Polyunsaturated fatty acids.

Ferroptosis inducers

Class I inducers such as erastin, sorafenib, sulfasalazine and glutamate, deplete cellular cysteine by inhibiting system Xc- and the biosynthesis of GSH, resulting in the loss of GPX4 activity[81,99-102]. The low water solubility and metabolic instability of erastin has limited its clinical application[103], but a metabolically stable erastin derivative has been tested[104].

Class II inducers, including RSL3 and DPI compounds, act by directly inhibiting GPX4[88,105-107], leading to the accumulation of lipid peroxides and eventual cell death. BSO and cisplatin also deplete GSH inducing ferroptosis. Cisplatin and erastin have a significant synergistic effect[108]. Interestingly, erastin promotes ferritinophagy and increased the free iron, lipid peroxidation, while RSL3 does not interfere with ferritinophagy, suggesting that RSL3 induction of ferroptosis is not dependent on ferritin degradation[109].

Class III inducers such as FIN56 act by both direct degradation of GPX4 and indirect inactivation of GPX4 *via* the squalene synthase-mevalonate pathway of the mitochondrial electron transport chain[103,110]. FIN56 also acts by depleting GPX4 and CoQ10. It seems that the cellular lethality of FIN56 is increased when cells are co-treated with statins and FIN56[110]. In addition, statins, such as simvastatin, enhance ferroptosis by inhibiting HMG-CoA reductase[110].

In class IV inducers, ferroptosis is induced by excess iron, omega-3 PUFAs, or peroxides, such as FINO2, that initiate lipid peroxidation and indirectly reduce GPX4 activity[111,112]. FINO2 is the only class IV ferroptosis inducer tested so far, but several other have been synthesized[103]. PUFAs show anticancer activity[113], but shortcomings such as reduced bioavailability, limited resistance to oxidative degradation, and lack of uptake specificity impede their use. However, the application of nanotechnology improves their therapeutic use[114]. Low density lipoproteins (LDLs) are taken up by LDL receptor expressed in tumor cells. LDL-based nanoparticles with docosahexaenoic acid (LDL-DHA NPs) were found to maintain their stability and specificity[115,116].

Experimental evidence suggests that there are additional biological inducers of ferroptosis, but their significance in human disease is still unknown. As mentioned above, ferritinophagy is a special recycling process of autophagy for the autophagic degradation of ferritin in lysosomes. It is mediated by the autophagic cargo receptor NCOA4, and leads to the initiation of ferroptosis[117]. Augmented ferritinophagy mediated by an increase of NCOA4 leads to induction of ferroptosis[64] (Table 1).

Reduction of iron-response element binding protein 2 significantly reduces erastin induced ferroptosis[55]. Increased activity of HO-1, the enzyme responsible for degradation of heme into ferrous iron, carbon monoxide, and biliverdin, increases LIP and initiated ferroptosis[118,119]. Artesunate (a derivative of artemisinin) is used in severe malaria[120] and induces hematopoietic stem cell (HSC) ferroptosis. However, the malaria drug chloroquine (a ferritinophagy inhibitor) reverses this effect, implying that artesunate induces HSC ferroptosis by activating ferritinophagy[121].

Finally, magnesium isoglycyrrhizinate (MgIG) is a natural product with anticancer activity[122] that has been shown to promote HSC ferroptosis. Inhibition of HO-1 reduces MgIG-induced HSC ferroptosis, suggesting that the promotion of HSC ferroptosis is mediated through upregulation of this enzyme[123].

LIVER MACROPHAGES IN IRON METABOLISM AND FERROPTOSIS

Kupffer cells and other liver and spleen macrophages take up heme from damaged or senescent erythrocytes and either export the extracted Fe^{2+} using ferroportin or store it in ferritin in the cytoplasm [124]. It has been shown that intracellular iron regulates the differentiation of macrophages into M1 (pro-inflammatory) and M2 (anti-inflammatory) subtypes[125,126]. M1 macrophages have an iron storage capability with higher HAMP but lower FPN and IRP1/2 compared to M2 subtype[127]. M1 polarization is regulated by iron overload[128], but also by ROS production and p53 acetylation induced by iron overload[129]. Recently, experiments with cultured macrophages demonstrated that chronic iron overload may in fact downregulate M1 markers and show signs of M2 differentiation[130].

During infection, hepcidin blocks macrophage differentiation to reduce iron export that could increase the growth of pathogens[131], which is reversed in the case of intracellular pathogens. This is possibly achieved by an increased production of nitric oxide[132] and the expression of the phagolysosomal protein NRAMP1 both leading to induction of ferroportin and intracellular iron reduction [133].

Kupffer cells exhibit phagocytic dysfunction and impair iron homeostasis during the development of non-alcoholic fatty liver disease (NAFLD)[134-136]. In addition, they participate in the clearance of lipids in nonalcoholic steatohepatitis (NASH) through M1 differentiation with the help of invariant natural killer T cells[137-139]. This composite role indicates that Kupffer cells can influence the development of ferroptosis, providing a new target for therapy in NAFLD.

Moreover, acute iron deprivation led to changes in metabolic and immunoregulatory genes in human macrophages resulting in impaired cell proliferation and reduced inflammation[140]. This is in contrast to the pro-inflammatory production of leukotrienes by the enzyme 5-LOX mediated by ferric iron in human macrophages[141]. As expected, ferroptosis has been the subject of several detailed reviews[69, 142-145], which include descriptions of ferroptosis regulators[146,147], ferroptosis in viral disease[148], and the role of macrophages in ferroptosis[149].

IRON IN LIVER DISEASE

Patients with chronic liver disease may exhibit hepatic and splenic iron loading, usually inside Kupffer cells and splenic and bone marrow macrophages[150]. Sometimes, this is accompanied by low hemoglobin levels and other hemolysis indices, indicating that hemolysis may have a role in the development of secondary iron overload[151]. However, a recent review emphasized the role of low levels of hepcidin in various liver diseases as implicated in both iron deposition in hepatocytes and participation in stellate cell activation and liver fibrosis[152].

Excess free iron exerts a toxic effect on the liver, favoring the progression of liver disease[58,153], and indeed abnormalities of iron regulation are reported in various liver diseases apart from inherited hemochromatosis[154]. Hyperferritinemia has been the main manifestation of disturbed iron homeostasis in chronic liver disease[155,156].

Opposing views have also been expressed in the literature. Data from cell culture experiments and animal models suggest that iron overload is only a weak fibrosis inducer and rarely causes serious liver damage not supporting the concept that iron overload is an important cause of liver toxicity. Iron may co-exist with other causes of inflammation, and the resulting hepatocyte necrosis is the real driving force leading to fibrosis[157].

The role of iron overload and the significance of ferroptosis have been investigated in the context of several liver diseases. The most common liver diseases will be discussed as well as the common end points of all, namely cirrhosis sometimes followed by the development of HCC. The rather limited available information on other liver diseases will be presented.

NAFLD/NASH

The role of iron in liver damage has been extensively researched in the case of NAFLD. A new term was introduced, the dysmetabolic or insulin-resistance hepatic iron overload syndrome (DIOS or IR-HIO), which is characterized by high serum ferritin levels, unexplained iron overload, and is associated with metabolic abnormalities[158-161]. IR-HIO is detected in one third to half of patients with NAFLD[155, 158,162,163]. The reason for the observed iron overload in NAFLD is still uncertain. A proposed mechanism is the redistribution of transferrin receptors (TfRs) to the cell surface, a process induced by insulin[158,163,164]. Tfr1 is upregulated in mice on a high fat diet, which may enhance hepatocellular iron uptake in NAFLD despite already increased hepatocellular iron[165]. The increase in serum ferritin may be due to increased iron stores, oxidative stress caused by lipid abnormalities, systemic inflammation, and genetics[166,167]. The implication of the presence of the Cys282Tyr *HFE* gene variant of hereditary hemochromatosis was also examined. A heterozygous mutation is associated with bridging fibrosis or cirrhosis in Caucasians[168-170]. By contrast, in knock out mouse models of hemochro-

matosis no progression to steatohepatitis or liver fibrosis was noted with a high-fat diet[171].

In addition, certain variants of ceruloplasmin are associated with increased liver iron stores and high ferritin in patients with NAFLD and advanced liver fibrosis[172,173]. Ceruloplasmin mutations have been associated with iron deposition in the liver of other chronic liver diseases as well[174]. Excess dietary iron causes hepatic oxidative stress, inflammation and hepatocellular ballooning injury leading to NASH[175,176]. Oxidative stress interferes with mitochondrial function, impairing fatty acid oxidation and producing different pro-inflammatory factors such as tumor necrosis factor (TNF)-α, IL-6, IL-8, MDA and nitric oxide[177-180], leading to NASH. Moreover, liver iron deposition increases cholesterol synthesis, lipid accumulation, and impairs cellular stress responses, which further exacerbate NAFLD[181-184].

The pattern of hepatic iron deposition is important in NAFLD patients, as iron deposition in macrophages is associated with more advanced disease[185]. An important observation was recently reported emphasizing the role of liver macrophages in the pathogenesis of NASH. A histological structure, the crown-like structure, has been described in NASH: Iron-rich Kupffer cells surround dead hepatocytes, take up debris, and induce inflammation and fibrosis. They have proinflammatory and profibrotic phenotypes, driving liver fibrosis[186]. Hepatic iron was significantly higher in patients with HCC associated with NASH and it was mostly localized in Kupffer cells[187]. Evidence suggests that iron may contribute to NAFLD pathogenesis and fuel the progression to NASH[178-180].

Red blood cell fragility and erythrophagocytosis may also explain iron deposition in NAFLD. It could be the result of insulin resistance and membrane lipid abnormalities[188]. Recently, aristolochic acid-associated drugs (atypical antipsychotic medications) were reported to induce NAFLD and link insulin resistance with iron metabolism dysregulation irrespective of drug-associated weight gain[189]. Regardless, whatever the etiology of the iron deposition in NAFLD and NASH, the clinical consequences are well documented.

Hyperferritinemia is also frequent in patients with NAFLD. Sometimes, it is the first laboratory abnormality leading to further clinical investigation[190]. In a large prospective population-based study from South Korea, serum ferritin was a strong early predictor of future development of steatosis, indicating that the ferritin association with NAFLD is not a simple consequence of the disease itself [191]. Patients with high ferritin have more severe steatosis[192,193], inflammation[194], advanced fibrosis[195], and increased mortality[196,197]. It has been suggested that serum ferritin could be used as a marker to identify NAFLD patients likely to have NASH and fibrosis[166]. However, a clear association between serum ferritin and fibrosis could not be verified in other studies that reported that ferritin could not accurately predict advanced fibrosis in NAFLD[198,199,200]. This discrepancy may be explained by the findings of a recent investigation in which hyperferritinemia was found in a quarter of NAFLD patients. In this study, stainable iron was present in hepatocytes, Kupffer cells, or more frequently, in both. Importantly, serum ferritin was not related to the presence of NASH, but it increased with worsening of fibrosis and decreased in the cirrhotic stage of the disease[201].

Iron measurement by magnetic resonance imaging (MRI) demonstrates that liver iron is the most important determinant of serum ferritin in NAFLD[202]. An important association of serum ferritin with the gut microbiome was also recently reported. In this study, ferritin levels were associated with differences in gut microbial composition. Both negative and positive associations with particular microbial species were found, and ferritin-related bacterial species correlated with hepatic iron-related genes. Moreover, the iron-associated microbiome was also linked to liver fat load. Fecal transplantation from high-ferritin mice to normal mice confirmed the human results and demonstrated an interplay among iron load, liver fat, and gut microbiome that could be exploited in future treatments[203].

Hepcidin in NAFLD

As in other liver diseases, extensive research has been conducted on the possible role of hepcidin in NAFLD. Investigations have tried to identify if the reported hepcidin abnormalities were the cause or the result of the iron overload observed in many cases of NAFLD. In various studies, hepcidin has been demonstrated to be either increased or decreased in NAFLD. In obese individuals, adipose tissue expression of hepcidin was upregulated, irrespective of steatosis and NASH. The contribution of adipose tissue hepcidin to the serum hepcidin is not well studied, but it may potentially explain the increased serum hepcidin in NAFLD[182,204-207].

Furthermore, leptin was found to correlate with hepcidin levels in obese children. Leptin also upregulates hepcidin expression in hepatocyte cultures, indicating that an increase in hepcidin may correlate to the leptin abnormalities in NAFLD[208,209]. Hepcidin downregulation, on the other hand, may be a consequence of oxidative stress secondary to iron overload[158-160,208,210]. Experimental evidence has demonstrated that hepcidin downregulation is a secondary phenomenon occurring after deposition of iron in the liver and the concomitant increase in oxidative stress[211]. Furthermore, an investigation on the relationship between iron stores and cardiovascular damage in patients with NAFLD showed that ferritin was associated with the components of the metabolic syndrome but not with liver inflammation and damage. In this study, hepcidin was increased due to the increased iron load[198], and fat in the liver of mice increased the expression of BMP-binding endothelial regulator, which was produced in sinusoidal endothelial cells and inhibited the BMP-SMAD pathway leading to a secondary inhibition of hepcidin. This is an additional explanation for the iron deposition in NAFLD

[212].

Clinical data also indicate that hepcidin abnormalities are not the primary cause of the excess iron in the liver observed in NAFLD. HJV levels were low and hepcidin levels were high in iron-overloaded NAFLD patients. These findings support the suggestion that iron accumulation may be the primary inciting event in this disease[213].

Individuals with the metabolic syndrome preserve the iron regulatory control of hepcidin, and hepcidin progressively increases in response to the increase of iron stores[205,214-216]. In addition, serum hepcidin and HAMP mRNA in the liver correlate to body iron stores irrespective of the degree of iron deposition. Thus, the dysmetabolic iron overload syndrome (DIOS) syndrome seen in NAFLD is not related to altered hepcidin synthesis[217].

However, despite the elevated serum hepcidin, duodenal iron absorption is increased because DMT1 is upregulated by IRP1 activation, likely due to unidentified humoral factors in the sera of NASH patients[218]. It seems, therefore, that elevated hepcidin in NAFLD is either a reflection of hepatocellular inflammation in NASH, or that increased iron and the associated induction of hepcidin appears before the development of NAFLD or NASH[219].

So far, data suggest that the interplay between iron and lipid metabolism is multifaceted in NAFLD. Moreover, it could be suggested that iron is directly implicated in NAFLD pathogenesis. Reports that increased dietary iron from red meat may predispose individuals to type II diabetes and insulin resistance are supportive evidence for such an idea[220-222].

Contrasting results have also been reported. For example, inadequate hepcidin production in response to a given level of iron load in NAFLD patients compared to controls was found in one study [159]. An impairment in the ability of hepcidin to inhibit iron absorption was also demonstrated in DIOS, suggesting hepcidin resistance in this condition[223]. The recent description of ferroptosis has prompted new investigations on the effects of liver iron load in NAFLD, although its exact role in this disease process has not been fully clarified.

Ferroptosis was recently related to the induction of inflammation in the early stages of NASH, making it a possible the “first hit” in its pathogenesis[98]. Further studies indicated that ferroptosis plays a critical role in the progression of NASH, making it a promising treatment target[224,225]. The enzyme arachidonate 12-LOX is known to promote the progression of NASH[226,227], and arachidonic acid metabolism has been shown to trigger ferroptosis in a diet-induced NASH mouse model[224]. Furthermore, the levels of the central regulator of ferroptosis ACSL4 were increased in a rat NASH model, and inhibition of the Mfn2/IRE1αACSL4 pathway was found to prevent incidence and development of NASH[228]. However, the connection between NAFLD and ferroptosis is still debatable, and many reviews of iron and NAFLD pathogenesis have been presented[229-231].

ALCOHOLIC LIVER DISEASE

Early reports showed that stainable iron is present in the livers of alcoholics[232,233]. Hepatocyte iron deposition is considered an important feature of alcoholic liver disease (ALD), although stainable iron in Kupffer cells is more prominent, particularly in the advanced stages of disease[234]. Ethanol consumption triggers iron overload[235]; it has been shown in patients with ALD that ethanol increases iron uptake from circulating de-sialylated transferrin by hepatocytes[236].

Almost half of patients with ALD have hepatic iron overload (HIO)[237] with high values of plasma ferritin and transferrin saturation[238,239]. Drinkers, from an early age, have increased iron markers [208,240]. High liver iron was found to be predictive of HCC development or death in patients with alcoholic cirrhosis[241,242], often acting synergistically with diabetes mellitus and viral hepatitis[243, 244]. Ethanol is metabolized into acetaldehyde, forming DNA and protein adducts that predispose individuals to HCC[103]. Iron is directly implicated in HCC development, since it accumulates in lysosomes through ferritinophagy and reaches the cytoplasm as free iron[245]. The resultant production of free radicals through the Fenton reaction initially activates Kupffer and stellate cells, ultimately leading to ferroptosis[246]. Additional significant production of ROS is mediated by cytochrome P450 2E1 (CYP2E1), which is directly induced by alcohol[247]. Alcohol consumption results in up to 20 folds increase of CYP2E1[248]. Additional mechanisms of alcohol-induced HCC have also been reviewed [249].

The question of increased iron load in the liver of patients with ALD has prompted research on hepcidin regulation in ALD. Suppression of hepcidin expression by ethanol has been reported in cell culture and experimental animal models, possibly *via* the inhibition of CCAAT enhancer binding protein-α (C/EBP-α)[250-253]. Iron induces activation of C/EBP-α, but ethanol inhibits this action and leads to inadequate hepcidin expression[254]. Suppression of the BMP6/SMAD pathway by alcohol has also been reported[255]. Hepcidin downregulation is also mediated by the induction of oxidative stress caused by either the effects of ethanol itself or free iron. As such, antioxidant treatment attenuates hepcidin downregulation[citation]. Ethanol may also increase hepatocyte iron uptake by upregulating the expression of TfR[246], even in habitual drinkers[256]. Additionally, ethanol may reduce hepcidin through proteins involved in liver regeneration; however, this requires further investigation[257].

Ethanol exposure simultaneously increases the expression of DMT1 and FPN in the duodenum[254], which has been linked to liver fibrosis[254,258]. Iron absorption is increased two-fold in chronic alcoholics[259], and ethanol administration in a mouse model overexpressing adipose tissue lipin-1 accelerated iron accumulation followed by lipid peroxidation, reduction of GSH, and induction of ferroptotic liver damage[260].

The effect of ethanol on hepcidin seems to be more complex than previously thought[261]. Ethanol has been shown to increase transforming growth factor (TGF)-β expression and phosphorylation of SMAD2[262]. Increased activation of SMAD2/3 can abrogate the TGF-β-induced hepcidin upregulation [263]. Hepcidin is also suppressed by ethanol through the toll-like receptor 4 (TLR4) pathway, and ethanol does not suppress hepcidin in TLR4 receptor mutant mice[264]. Interestingly, TLR4 deficiency has been shown to protect animals from liver fibrosis[265,266]. Further evidence suggests that ethanol action on TLR4 involves HSCs, as TLR4 on Kupffer cells or mature hepatocytes are unlikely targets of the effects of ethanol[267,268].

Both serum transferrin and serum hepcidin have been used as prognostic markers in ALD. To this end, low transferrin levels[269,270] have been associated with worse prognosis[197,270-272]. Importantly, the prognostic value of serum transferrin is similar to other traditional prognostic scores, such as the model of end-stage liver disease (MELD) and the Glasgow alcoholic hepatitis scores[273].

The recent identification of ferroptosis has allowed for a better understanding of the connection between lipid and iron abnormalities observed in ALD[274]. Ferroptosis is downregulated during the repair of ethanol-induced liver damage, while ferroptosis inhibition or activation of the Nfr2 pathway reversed ROS accumulation and lipid peroxidation induced by ethanol[275,276]. Excessive ethanol activates genes like frataxin that promote liver injury[277]. More importantly, ferroptosis provides a strong link for the recently demonstrated crosstalk between the liver and the gut[278]. Lack of intestinal sirtuin 1 has been shown to limit ferroptosis, normalize iron overload, and ameliorate ethanol-induced liver damage[279]. Ferroptosis is also implicated in adipose-liver axis abnormalities observed in alcoholic steatohepatitis[260]. Moreover, the overexpression of adipose-specific lipin-1 aggravates alcoholic steatohepatitis and iron deposition, increasing hepatic MDA levels[153,260].

Finally, an additional mechanism of ethanol-induced liver damage has been identified in severe alcoholic hepatitis patients. Iron overload triggers activation of the metallopeptidase ADAM17, which leads to the increase of TNF-α and soluble CD163, resulting in macrophage activation and promotion of hepatic inflammation[280]. Detailed reviews on iron and ALD were recently published[143,281,282].

CHRONIC HEPATITIS C

The effect of iron on the activity and infection cycle of the hepatitis C virus (HCV) has been controversial. Inhibition of viral replication by iron due to the suppression of the nonstructural protein 5B has been reported[283], but enhancement of viral replication has also been observed[284]. HCV alters the expression of hepcidin and therefore cellular iron metabolism[285,286]. Experimental evidence in early HCV infection has demonstrated increased hepcidin expression followed by enhanced viral translation and replication. In this study, iron loading of macrophages accompanied hepcidin upregulation and resulted in increased viral transmission to naïve cells[287]. Other experimental studies, however, have shown that hepcidin levels are low in HCV-infected cell lines[288,289].

Inhibition of hepcidin expression has been attributed to HCV-induced oxidative stress[290,291]. Experiments in chimpanzees on high iron diets have demonstrated that liver damage is observed only in animals infected with HCV, indicating a harmful effect of iron in HCV infection[292]. In chronic infection, HCV interferes with the expression of the iron uptake receptor TfR1, a known mediator of HCV internalization[293,294]. The observed downregulation of hepcidin despite hepatic inflammation in chronic HCV[295] may be related to impairment of the BMP6/HJV pathway by TNF-α, which would suppress the transcription of HJV[296].

Clinical studies have verified that HCV infection downregulates hepcidin[297-299], and serum hepcidin has been correlated with severity of liver disease[300]. More than 40% of patients have iron overload associated with a high rate of liver damage and inflammatory activity, as well as an increased risk of hepatocarcinogenesis[301-303]. Hepatic iron and HCV proteins in combination produce a toxic hydroxyl radical ($^{\cdot}$OH) that forms mutagenic bases such as 8-hydroxy-2-deoxyguanosine (8-oxodG)[304, 305]. HCV patients have been shown to have an approximately 10-fold increase of 8-oxodG in liver tissue compared to non-HCV control patients[306].

The Hepatitis C Antiviral Long-Term Treatment Against Cirrhosis trial has convincingly demonstrated that iron in hepatocytes and portal tract cells predicts progression to decompensated cirrhosis, HCC, and death[307]. Almost all liver tissue from HCV patients had some lysosomal iron deposits detected by electron microscopy and X-ray microanalysis, despite negative results with classical Prussian Blue staining[308]. Moreover, increased serum aminotransferases were found only in HCV patients with stainable iron in Kupffer cells but not in those with hepatocellular iron[309].

Even minor increases in iron load in heterozygous carriers of C282Y or H63D gene mutations for hemochromatosis were found to induce more fibrosis in chronic HCV infection[310,311]. Genotype 3-

infected patients have more frequently elevated liver iron, which has been associated with hepatic steatosis in this type of HCV infection[312]. Evidence from thalassemia patients further indicates that iron adversely affects the disease course of HCV, increasing morbidity and mortality due to more severe liver disease[313].

Liver iron also adversely affects the response to interferon (IFN)-based treatments[314]. In studies involving IFN treatment, ferritin levels increased regardless of sustained virologic response (SVR) and decreased at about 3 years post-treatment. This is not the case with direct-acting antivirals (DAAs)[315,316], where SVR is achieved irrespective of iron status[317-320]. A recent study demonstrated that pre-treatment elevated serum ferritin and ERFE levels were restored after treatment with DAAs and correlated with changes in LDL cholesterol levels, but only in men[321].

Plasma ferritin, liver iron, and transferrin saturation are also increased in HCV infection[322] and elevated serum ferritin has been related to liver fibrosis[323]. An additional reason for increased liver iron in HCV patients is the reported increased hemolysis particularly in advanced stages of the disease [151]. Despite the evidence presented above, different results in relation to the role of HCV-induced iron overload have been presented[324,325]. In several studies, elevated serum ferritin and hepatic iron played no significant role in the progression of liver damage[326,327]. Moreover, the significance of hemochromatosis mutations has been questioned as a risk factor in the progression of HCV-related disease[328].

Recently, it has been suggested that ferroptosis may be implicated in the natural course of HCV[58]. Importantly, HCV replication is inhibited by an iron-dependent mechanism like ferroptosis, which is mediated by the desaturation of oleate to highly unsaturated fatty acids by the enzyme fatty acid desaturase 2 (FADS2). This is a key determinant of cellular sensitivity to ferroptosis; FADS2 suppression significantly enhances HCV replication, whereas the ferroptosis inducer erastin sensitizes HCV to DAAs, altering the conformation of HCV replicase[329].

CHRONIC HEPATITIS B

Iron favors hepatitis B virus (HBV) mRNA expression in HepG2 cells[330]. Increased serum and cellular iron uptake and decreased hepcidin expression have been reported in HBV infection[297,331]. Hepatitis B-infected patients frequently show iron deposition in hepatocytes and elevated liver iron concentration (LIC) leading to increased disease severity[332,333]. Serum ferritin levels are also increased in patients with chronic HBV[332].

Levels of hepcidin are increased in early stages of HBV and reduced in the cirrhotic stage[334,335]. Co-infection with hepatitis D increases the iron load[332]. However, results of studies regarding serum hepcidin in HBV infection are not uniform. Reduced serum hepcidin has been reported in HBV patients with or without cirrhosis[336], while another report found that hepcidin is slightly increased in HBV patients without cirrhosis and in those with HCC[335]. The reason for this discrepancy is not clear. Nonetheless, decreased hepcidin levels and elevated transferrin saturation and ferritin levels have been associated with fibrosis severity in patients with chronic HBV[337].

It should be noted that iron deposition in the liver has been considered a secondary phenomenon. Damaged hepatocytes in viral hepatitis undergo necrosis and the released iron is scavenged by Kupffer cells[240,338]. However, this mechanism cannot entirely account for the deposition of iron in hepatocytes. The implication of HBV in iron deposition is exemplified in a case report in which a female patient with symptoms of iron overload had highly increased serum ferritin and transferrin saturation. In this case, all of the patient's symptoms resolved and her iron abnormalities normalized after HBV antiviral monotherapy[339].

LIVER FIBROSIS AND CIRRHOSIS

Nearly 6 decades ago, it was shown that iron on liver biopsy is associated with manifestations of advanced disease compared to that in non-iron overloaded cirrhotic patients[340]. Cirrhotic patients with hemosiderosis are more likely to be classified as Child Pugh class B or C with higher MELD scores than those without stainable iron[341,342].

As mentioned before, hyperferritinemia and high liver iron predict the risk of advanced liver fibrosis in NAFLD[166,179,343]. A recent study of a large number of NAFLD patients with a long follow-up (mean 8.4 years) emphasized the fact that it is the non-parenchymal iron deposition that leads to serious liver disease[344].

Fibrosis is increased by the presence of iron through increased HSC proliferation and selectively increased collagen synthesis without interference by non-collagen proteins[345,346]. Experiments with cultured HSCs have shown that incubation with either ferritin or transferrin increases nuclear factor kappa-B translocation and HSC activation[347,348], and enhances α-smooth muscle actin, collagen, and vimentin synthesis[349]. Isoprostanes, products of arachidonic acid peroxidation produced during iron-induced oxidative stress, increase HSC-collagen-production and TGF-β release from Kupffer cells[350].

Furthermore, 4-HNE upregulates the expression of collagen and the TIMP-1 inhibitor of metalloproteases in HSCs[351].

Elastin, another component of the extracellular matrix, is also affected by iron. Elastogenesis is modulated in cultured human skin fibroblasts by iron, as evidenced by the levels of both elastin protein and elastin mRNA are increasing 3-fold[194]. Liver iron load also induces both TGF-β[352] and BMP-6 [353,354]. The connection between fibrosis and hepcidin pathways and the significance of SMAD4 as their common link has been demonstrated[353]. Other signaling pathways related to fibrosis are also modulated by iron. For example, iron deficiency stimulates Notch signaling[355], and recently, iron-loading revealed a protective role of β-catenin (a component of the cadherin complex that stimulates Wnt signaling) against liver fibrosis[356]. Hepcidin also has a protective role in liver fibrosis by suppressing HSC activation[357]. BMP6, the main hepcidin inducer, has a similar protective role in fibrosis inhibiting HSCs activation[358]. Evidence regarding the role of ferroportin in liver fibrosis is limited. However, ferroportin has been shown to be increased in activated HSCs and the anti-fibrotic action of hepcidin in HSCs mentioned above may be mediated by degradation of ferroportin[357].

Clinical evidence confirms the importance of iron metabolism in the development of fibrosis. For example, ferritin levels have been associated with decompensation and increased mortality in cirrhosis [359]. However, ferritin concentration has poor sensitivity as a marker of liver fibrosis, since it also increases as a result of inflammation[360]. Transferrin also has clinical significance in HCV- and HBV-related cirrhosis; it has been associated with advanced fibrosis and is a predictor of survival in cirrhotic patients[269,301,338]. Additionally, low hepcidin levels can cause iron overload and increased oxidative stress in the liver[361], which in combination with other factors such as genetic variables, viral infections, and alcohol use, can eventually lead to liver fibrosis[362].

Low hepcidin has also been demonstrated as a predictor of mortality and development of HCC in alcoholic cirrhosis[258,363]. Similarly, in HBV cirrhosis, hepcidin is low compared to patients without cirrhosis[335,364], where values are similar to healthy controls[335,365]. In HCV-related cirrhosis and alcoholic cirrhosis, hepcidin is significantly lower than in HBV cirrhosis[365-367].

Hepcidin levels are not reduced in the early stages of NAFLD, but eventually drop in advanced fibrosis, similar to what has been observed in other liver diseases[368]. Unlike ferritin[369], serum hepcidin is a reliable marker of severity of fibrosis in NAFLD[368,370]. A low hepcidin/ferritin ratio can differentiate between cirrhosis and non-cirrhosis in patients with HBV, HCV and NAFLD[367], but not in ALD patients, possibly because ethanol directly inhibits hepcidin expression as mentioned above.

Ferroptosis

The role of ferroptosis in liver fibrosis was recently investigated. Its role is debatable as both induction and attenuation of liver fibrosis by ferroptosis has been reported. Ferroptosis increased susceptibility to fibrosis in mice on a high-iron diet, an effect reversed by a ferroptosis inhibitor[12]. However, other studies have shown that ferroptosis attenuates HSC activation and reduces liver fibrosis. Moreover, the ferroptosis inducers erastin and sorafenib reduce liver fibrosis increasing ferritinophagy[371], and MgIG increases ferroptosis, leading to reversion of fibrosis[123]. The anti-malarial agent artemether increases the p53-dependent ferroptosis and inhibits HSC activation[372] and artesunate, a derivative of artemisinin with immunomodulating properties, induced ferroptosis of activated HSCs possibly triggering ferritinophagy[121]. The role of iron in liver fibrosis has been recently reviewed[152,373].

HCC

Hepatic iron overload has long been linked to HCC tumorigenesis and tumor growth[147,374-376]. Iron incubation of an HCC cell line has been shown to increase mesenchymal and metastatic markers, representing a fundamental defect in cancer development[377]. Patients with hereditary hemochromatosis show a 20-200-fold increase risk of HCC development[378,379]. Additionally, iron score has been demonstrated to be significantly higher in HCC-NASH patients than in NASH controls[343]. In HCC patients, iron localization is mainly sinusoidal[187], and iron deposition in the portal tract has been associated with poor survival after tumor resection[380]. Similar findings have been reported in prospective studies of HCC in alcoholic cirrhosis[241] and in HCV-associated cirrhosis[381].

Several studies suggest an association between HCC and dietary iron overload from beer fermented in steel drums in black Africans[382-385]. Furthermore, experimental evidence has identified several mechanisms of iron involvement in HCC development. Namely, HCC cells, like many other cancer cells, upregulate iron uptake and intracellular iron accumulation since they are dependent on iron[386,387]. The generation of ROS by this iron favors carcinogenesis through promotion of genomic instability and generation of DNA repair defects[388,389]; in other words,, this generation of ROS maintains the oncogenic phenotype of cancer cells[390,391]. The direct hepatocarcinogenic effect of free iron in the pathogenesis of HCC has also been demonstrated in an animal model of iron-rich diet where the tumor developed without fibrosis or cirrhosis[392,393]. Additionally, iron deposition directly decreases p53 protein level and its activity in the liver, facilitating the development of HCC[394]. An important mediator of intracellular iron is the protein leucine-rich repeat protein 5 (FBXL5); exposure of FBXL5

knockout animals to chemical or viral carcinogens has been shown to result in increased liver tumor formation. More importantly, low levels of FBXL5 in HCC patients are associated with a poor prognosis [395]. Ferritin heavy chain (FTH) acts as a protector of HCC cells, increasing their cellular resistance to ferroptosis, thereby acting as an oncogene in the pathogenesis and progression of HCC[396].

HCC patients in contrast to those with other cancers have low hepcidin levels[397-399]. Many mechanisms lead to the final decrease of hepcidin in HCC, including downregulation of inducers such as HAMP, TfR and HJV, and upregulation of suppressors such as matriptase 2 and GDF15[400]. Hepcidin downregulation increases cellular proliferation and HCC risk *via* reduction of the hepcidin protection against HSC activation. The downregulation of hepcidin in HCC has been attributed to the effects of cirrhosis rather than to HCC itself. Cirrhotic patients also show decreased hepcidin expression irrespective of disease etiology[152,336,399], while the hepcidin:ferritin ratio has been reported to decrease with fibrosis progression[373].

Ferroptosis and its inducers have been extensively investigated in HCC as it is considered an effective tumor suppression mechanism[81,401-403]. On the other hand, genes negatively regulating ferroptosis increase HCC drug resistance[404]. Sorafenib, a drug used for treatment of advanced HCC, is one example. This drug can induce the expression of metallothionein-1G (MT-1G), and upregulation of MT-1G has been demonstrated to serve as a negative regulator of ferroptosis, conferring resistance to sorafenib[405]. Some studies have also found that haloperidol can facilitate the cascade of ferroptosis induced by sorafenib in HCC[406].

In contrast to the negative regulators of ferroptosis, ACSL4 can positively regulate ferroptosis in HCC [97]. Inhibition of ACSL4 protects sorafenib-induced ferroptosis in HCC cells. A human study demonstrated an upregulation of the ACSL4 protein in HCC tissue from surgical specimens with a good response to sorafenib as a postsurgical adjunct treatment[407]. ACSL4 may therefore serve as a prognostic factor for survival and disease-free survival time[407,408].

Natural omega-3 PUFAs are the main peroxide substrates in ferroptosis and have anti-tumor activity [409], a fact that has been therapeutically exploited[410]. PUFAs consumed in the form of fish can reduce the risk of HCC development[411]. Ceruloplasmin has also been shown to inhibit ferroptosis in HCC cells, interfering with iron metabolism. Moreover, inhibition of ceruloplasmin increases the accumulation of iron and ROS production, facilitating erastin-induced ferroptosis in HCC cells[412].

Additional regulators of ferroptosis in HCC are the long non coding RNA molecules (lncRNAs), but their role has not been fully elucidated[413]. Erastin-induced ferroptosis upregulates the lncRNA GABPB1-AS1 in HepG2 cells, silencing the gene encoding peroxiredoxin-5 peroxidase and eventually leading to a reduction in cellular antioxidant capacity[414]. The predictive value of lncRNAs associated with ferroptosis in HCC has been recently addressed. Nine and five ferroptosis signature models have been established, which identified two groups of patients; the high-risk group in this study was shown to have enhanced tumorigenesis and worse prognosis[415,416].

Equally, the non-coding circular RNAs (circRNAs) seem to play a role in the development of HCC through ferroptosis. The circ0097009 endogenous RNA regulates the expression of SLC7A11, a key regulator of cancer cell ferroptosis in HCC. Circ0097009 therefore may be used as a potential target for HCC treatment[417].

Ferroptosis-related genes (FRGs) have also been identified and found to be upregulated in HCC tissue. In one study, three clusters have been determined, and a high expression of cluster 3 has been associated with worse prognosis and a higher histological stage[418]. Another approach regarding the use of ferroptosis as a prognostic marker in HCC has also recently been presented in which a novel ferroptosis-related 10-gene signature stratified HCC patients into two risk groups[419]. Those in the high-risk group have significantly reduced survival. The role of ferroptosis in HCC generation and progress has been recently reviewed[420].

CHOLESTATIC DISEASES

Hepcidin is significantly lower in patients with primary biliary cholangitis and primary sclerosing cholangitis compared to patients with other chronic viral and metabolic liver diseases. In one study, low hepcidin was maintained even after two years of treatment[421]. The reason for low hepcidin may be the suppression of STAT3 phosphorylation by accumulated bile acids. Furthermore, hepcidin remains lower in cholestatic cirrhosis compared to non-cholestatic cirrhosis, suggesting the critical role of cholestasis in maintaining low values of hepcidin[422].

AUTOIMMUNE HEPATITIS

There is experimental evidence suggesting that iron is implicated in autoimmune hepatitis (AIH) through ferroptosis involvement. The classical AIH-inducer Concanavalin A (ConA) has been linked to an overproduction of reactive nitrogen species (RNS) such as nitric oxide and peroxynitrite in a mouse model of AIH. This effect is attenuated by Fer-1, indicating that ConA induces ferroptosis in the liver.

Moreover, gadolinium chloride (a Kupffer cell depleting agent) inhibits RNS and hepatocyte ferroptosis [423]. Indoleamine 2,3-dioxygenase 1 (IDO1) is an intracellular heme enzyme involved in autoimmune diseases[424]. Upregulation of IDO1 has also been shown to be involved in ConA-induced hepatocyte ferroptosis through RNS accumulation and hepatocyte ferroptosis. An IDO1 inhibitor and an IDO1 knockout were shown to induce this effect, indicating that IDO1 promotes hepatocyte ferroptosis by triggering nitrative stress[425].

Clinical evidence also supports the detrimental effect of iron in AIH. Ferritin and iron are increased in serum of 65% and 58% of naïve patients with AIH respectively, which is resolved after successful treatment[426]. Increased serum ferritin has been independently associated with advanced fibrosis in patients with untreated AIH[427]. Moreover, serum hepcidin is low in patients with liver autoimmune disease[367,421]. Interestingly, in AIH, low serum hepcidin levels remain after 2 years of treatment, a finding similar to observations in autoimmune cholestasis. A plausible explanation could be that hepcidin is involved in hepatic autoimmune processes[428].

ISCHEMIA-REPERFUSION INJURY

Although ischemia-reperfusion injury (IRI) is not strictly a liver disease as it also occurs with other organ transplantations, iron is clearly involved in the pathogenesis of IRI-related hepatic abnormalities. Ferroptosis is implicated in the pathogenesis of IRI through GPX4 inactivation[59,429]. Iron overload and upregulation of the ferroptosis indicator PTGS2 are prominent characteristics of IRI in the liver[59]. An analysis of 202 live-donor liver transplantation patients showed a high serum ferritin level indicating iron overload[430]. In this study, use of ferroptosis inhibitors such as Fer-1, α-tocopherol, and DFO prevented hepatic IRI.

ACUTE LIVER FAILURE

Ferroptosis is also involved in the development of acute liver failure (ALF). In sepsis-induced ALF, analysis of the liver infiltrate has shown that FRGs may be responsible for the development of liver failure through the activities of B cells and natural killer cells[431]. The most common reason for ALF, however, is acetaminophen (APAP) toxicity in which lipid peroxidation leads to hepatocyte ferroptosis [432].

GSH is important for the inactivation of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) responsible for APAP toxicity. GSH reduction and GPX4 inhibition are common in APAP-induced cell death[433]. The viability of mouse hepatocytes in the presence of APAP is improved by fer-1 without restoring the cellular GSH level, suggesting that suppression of the conversion of APAP to NAPQI is not the reason for the protective effect of fer-1[434]. Consistently, other experiments have confirmed the role of ferroptosis in APAP-induced hepatocyte cell death[432,435-437]. An additional mechanism of APAP-induced ferroptosis is the significant hepcidin reduction, likely *via* activation of HIF1α[434,438-440].

However, the role of ferroptosis in APAP toxicity and other drug-induced liver injury is disputed. An earlier report showed that α-tocopherol does not improve APAP-induced liver injury and that lipid peroxidation is not involved in APAP hepatotoxicity[441]. A recent review suggests that APAP-induced hepatotoxicity should be identified as programmed necrosis and not ferroptosis or other types of cell death[442]. Therefore, more research is required before ferroptosis inhibitors are recommended as treatments for APAP toxicity.

SICKLE CELL LIVER DISEASE

Sickle cell liver disease (SCD) is an inherited disease caused by the presence of hemoglobin S. Under hypoxic conditions, red blood cells are dehydrated and form the characteristic sickle cells[443,444]. The formation of hemoglobin S is due to a single substitution of an amino (glutamic acid to valine) in the beta globin chain[444]. Viral hepatitis and iron overload are two major reasons for the development of liver disease in SCD, both of which are typically related to patients receiving multiple blood transfusions[445]. Sources of hepatic iron in SCD include these multiple blood transfusions and chronic intravascular hemolysis[446]. Liver iron deposition occurs mainly in Kupffer cells[447]. Liver iron deposition can also occur in non-transfusion dependent patients[448], and there is a single case described in a patient who never received any blood transfusion[449]. Hemosiderosis in SCD may lead to fibrosis and overt cirrhosis[445,448,449].

CORONAVIRUS DISEASE 2019

There is considerable evidence to suggest an association between ferroptosis and coronavirus disease 2019. Cytokines produced during the infection have been shown to upregulate hepcidin expression, which leads to ferroportin suppression and iron accumulation. In addition, severe acute respiratory disease coronavirus 2 downregulates the expression of GPX4, contributing further to the initiation of the Fenton reaction and production of massive amounts of ROS and associated ferroptosis[450].

TARGETING IRON

There have been many attempts to reduce iron overload, which is uniformly considered detrimental in liver disease irrespective of etiology. However, it should be remembered that iron loading is not always similar between patients and between stages of various diseases[152]. Dietary iron restriction has been shown to be effective in reducing liver fibrosis and steatosis in diet-induced NAFLD animal models[451,452].

Phlebotomy is the traditional treatment in hereditary hemochromatosis, as it increases erythropoiesis, partially reverses liver fibrosis, and increases life expectancy[453,454]. Phlebotomy has been used to treat NASH patients, but the clinical benefit is unclear[455]. Phlebotomy improves liver enzymes, insulin resistance, and liver histology in the majority of NAFLD patients, but it is not fully successful in DIOS insulin resistant patients with slight ferritin increase[182,456-458]. Insulin sensitivity is improved by phlebotomy in type II diabetics with a high serum ferritin[459]. Moreover, in patients with the metabolic syndrome, phlebotomy improves metabolic parameters, including glycosylated hemoglobin A1c and LDL/high-density lipoprotein ratio[460]. In a meta-analysis of four interventional studies with more than 400 patients, phlebotomy was shown to improve liver enzymes, insulin resistance, and lipid abnormalities[461].

In contrast, no effect was reported in two prospective randomized controlled trials. The first, which is the largest series so far, was conducted in NAFLD patients[462], and the second in DIOS patients with insulin resistance[463]. To this end, the benefit of phlebotomy in patients with NASH remains unclear until more extensive studies are available[464].

Phlebotomy reduces the marker of oxidative stress 8-hydroxy-2′-deoxyguanosine in HCV patients who have failed IFN therapy. Fibrosis and inflammation are also reduced, but HCV titers are unaffected. None of the patients in these studies were shown to develop HCC at the six year follow-up point[306,465]. Reduction of HCC development in HCV patients after phlebotomy has been verified in additional studies[466,467]. Phlebotomy has also been reported to improve the response to IFN in chronic HCV[468].

Iron chelation is an additional intervention to reduce liver iron. DFO has been successfully used to control fibrosis in hemochromatosis[469]. Studies in several animal models have revealed that iron chelation decreases the stability of procollagen mRNA[470] and reduces elastin mRNA[194]. DFO has also been shown to reverse HSC activation and induces apoptosis of activated murine HSCs[471]. More recently, a study of the combination of DFO with pegylated IFN-α showed a synergistic anti-fibrotic effect in rats[472]. ROS degrade the apolipoprotein B100 (apoB100) component of VLDL, thereby enhancing hepatocyte steatosis in rodents. In another study, DFO restored apoB100 and increased VLDL secretion[473]. No firm conclusions can be drawn, however, without the results of clinical trials. It should be noted that inhibition of hemoxygenase-1 decreases hepatic iron deposition and attenuates liver fibrosis in rats[474].

Interestingly, commonly used drugs like the calcium channel blockers have been found to induce HSC apoptosis and reduce DMT1 expression, hepatic iron deposition, and liver collagen in mouse and cellular experiments[475]. Hepcidin may be a promising agent for the treatment of liver iron overload, as hepcidin administration has been shown to attenuate iron deposition in mouse models of hemochromatosis[476-478], while its overexpression ameliorates fibrosis severity. This is due to the inhibition by hepcidin of the TGFβ1-induced SMAD3 phosphorylation in HSCs, a pathway that requires the presence of ferroportin in stellate cells[357]. Similar reduction of liver fibrosis has been observed with BMP6 overexpression in murine and human NAFLD[358].

Hepcidin responds to iron conditions in HCV patients, but the response is impaired. Thus, correction of hepcidin regulation may improve the clinical progress in iron-overloaded HCV patients[479]. Hepcidin manipulation may be beneficial in the management of HCC as well. The iron chelator deferasirox induces apoptosis in hepatoma cells lines and decreases liver tumor development in mice, increasing HAMP mRNA expression. However, toxicity and the lack of response in some patients may be a problem in human trials[480]. Additionally, some HCC patients have increased hepcidin expression and downregulation of hepcidin may be required. In a murine HCC model with high liver hepcidin, the traditional Chinese medicinal herb dandelion polysaccharide has been shown to reduce hepcidin expression, arrest the cell cycle, and suppress the HCC proliferation[481]. Hepcidin, therefore, is a logical candidate target for clinical trials in HCC. Indeed, both hepcidin agonists and inhibitors have been tested *in vitro* and in laboratory animals[482]. It should be noted that synthetic mini-hepcidins have

also been tested in Hamp -/- mice; in one study, serum iron was reduced after chronic administration of the drug[476].

Ferroptosis is the current therapeutic target in the treatment of iron overload diseases. It should be stressed, however, that the effects of ferroptosis in chronic liver disease depends on the cell type and the specific environment. In liver fibrosis, for example, ferroptosis has different effects on hepatocytes and HSCs as will be detailed later[483]. A future challenge is to develop drug delivery systems targeting ferroptosis in specific cell types. In ALD and in NAFLD, ferroptosis is implicated in liver damage, and ferroptosis inhibition would theoretically be beneficial[225,276,432]. For example, ferroptosis-induced liver injury could be reversed by sestrin 2, an antioxidant protein increased by ferroptosis inducers[484].

Ferroptosis inducers

In contrast to other liver diseases where ferroptosis is detrimental and therapies are directed towards inhibition of ferroptosis, HCC is benefited by enhancement of ferroptosis. Thus, ferroptosis inducers are used in advanced HCC. Sorafenib, a multi-kinase inhibitor, is the most extensively studied ferroptosis inducer[103,485]. In HCC, this drug acts by inhibiting cellular proliferation and neo-angiogenesis. Additionally, it induces ferroptosis in HCC cells[486]. It has been reported that sorafenib decreases the uptake of cystine in the Xc- system and starts the chain of events leading to ferroptosis induction through the accumulation of ROS, which is the result of GSH depletion and loss of GXP4 activity[487]. Excessive ROS production also results in the inhibition of the retinoblastoma protein Rb, an important negative regulator of cell proliferation[488].

Prolonged administration increases the resistance of HCC cells to sorafenib. ABCC5, a recently described regulator of ferroptosis, increases the generation of GSH and reduces the production of ROS through stabilization of SLCA11 and subsequent inhibition of ferroptosis. Accordingly, downregulation of ACCC5 reduces resistance to sorafenib[489]. Other proteins reducing the sorafenib-induced ferroptosis through stabilization of SLCA11 have also been recently described[490,491].

Haloperidol has also been shown to promote erastin- and sorafenib-induced ferroptosis, suggesting that it could be used in combination with sorafenib to achieve either dosage or resistance reduction[404,406,492]. An upregulation of Nrf2 through activation of the p62-Keap1-Nrf2 pathway inhibits sorafenib-induced ferroptosis in HCC cell lines[63,493]. Interestingly, trigonelline, the active ingredient of the traditional Chinese medicine fenugreek, increases ferroptosis by acting on Nrf2, therefore reducing sorafenib resistance[494]. Overexpression of the leukemia inhibitory factor receptor (LIFR) has also been shown to increase sorafenib-induced ferroptosis of HCC cell lines, whereas reduced LIFR expression increases resistance to ferroptosis[495].

A recent study reported an another target for HCC treatment. In this study, lactate-rich hepatoma cells were shown to exhibit increased resistance to the ferroptosis generated by common ferroptosis inducers. Moreover, lactate uptake was shown to be mediated by monocarboxylate transporter 1 (MCT1), which enhances the production of monounsaturated fatty acids, blocking ferroptosis. Inhibition of MCT1-mediated lactate uptake enhances ferroptosis[496]. In contrast to the presented evidence, a recent report indicated that sorafenib may not be an inducer of ferroptosis at least in many cancer cell lines[497]. Other drugs that could be used in the treatment of HCC based on increased ferroptosis have also been recently described[498,499]. Heteronemin, a marine terpenoid, induces ferroptosis in HCC cells by reducing GPX4[500]. IFN-γ has also been confirmed to inhibit system Xc- activity and increase ferroptosis[501]. Lenvatinib, another kinase inhibitor used in advanced HCC treatment, also acts through the inhibition of the system Xc-. Fibroblast growth factor receptor-4 (FGFR4) increases the activity of the system Xc- and lenvatinib inhibited FGFR4 increasing ferroptosis. Interestingly, patients with HCC positive for FGFR4 have a longer progression-free survival compared to those with FGFR4-negative HCC. Nrf2 upregulation has also been shown to decrease the sensitivity of HCC to lenvatinib [502]. Moreover, low-density lipoprotein nanoparticles (LDL-DHA NPs), selectively induce HCC cell death in mouse models, and LDL-DHA NPs enhance lipid peroxidation due to both GSH depletion (leading to GPX4 inactivation) and direct degradation[410].

Ferroptosis can be used for stratification of HCC patients to predict both prognosis and suitability for immunotherapy. For that purpose, a ferroptosis-related prognosis risk score model has been developed to stratify patients into two subgroups based on six FRGs (FRGs)[503].

Ferroptosis inhibitors are promising drugs in the treatment of various liver diseases, although evidence is mainly based on laboratory data. NAFLD and NASH progress is worsened by induction of ferroptosis[98,224,504]. Alleviation of NASH can be achieved by ferroptosis inhibitors, such as liproxstatin-1 or ferrostatin-1[225,505]. In one study, administration of the ferroptosis inducer RSL3 aggravated hepatic steatosis and inflammation in diet-induced NASH mice, while administration of liproxstatin-1 ameliorated NASH severity and rescued animals from cell death[225].

Other drugs, such as Ginkgolide B and dehydroabietic acid, alleviate NASH severity by inhibiting ferroptosis *via* upregulation of the p62-Keap1-Nrf2 pathway[506-508]. Thymosin β4 (Tβ4) improves liver lipid metabolism markers in NAFLD rat models and inhibits the palmitic acid-induced hepatocyte death in the LO2 cell line. Ferrostatin-1 increases the effect of Tβ4, which is attenuated by erastin, indicating that the protection of hepatocytes is mediated by ferroptosis reduction[509]. The enzyme enoyl coenzyme A hydratase 1 (ECH1) is an important component of mitochondrial fatty acid β-oxidation. ECH1 knockdown aggravates liver inflammation and fibrosis in mouse NAFLD models

while fer-1 administration alleviates liver damage, again suggesting that the beneficial effect of ECH1 may be due to inhibition of ferroptosis[505].

Liver fibrosis is another disease that may be treated by ferroptosis regulators[510]. Inhibition of ferroptosis by ferrostatin 1 reverses liver fibrosis induced by a high-iron diet or and carbon tetrachloride [511], while induction of ferroptosis by liver iron overload aggravates APAP-induced fibrosis in mice [483]. However, ferroptosis is a double-edged sword in liver fibrosis. When ferroptosis is targeting activated HSCs, the induction of ferroptosis is beneficial. The cystine/glutamate antiporter SLCA11 has been shown to increase ferroptosis as mentioned before[55]. Inhibition of SLC7A11 enhances ferroptosis in HSCs and attenuates liver fibrosis[512]. Likewise, erastin and sorafenib induce ferroptosis in HSCs, and reduced liver fibrosis in mice[371,513].

There is growing evidence that natural products may effectively be used in the treatment of liver fibrosis. Artesunate can attenuate liver fibrosis by triggering ferritinophagy-mediated ferroptosis in HSCs[121]. Artemether can also induce ferroptosis in HSCs by increasing iron and ROS in HSCs[514] and promoting p53-dependent ferroptosis[372]. MgIG can also induce ferroptosis in HSCs by increasing the activity of the enzyme HO-1[123].

Chrysophanol isolated from the rhizome of rhubarb can inhibit the HBV x protein-induced activation of HSCs through ferroptosis and alleviate HBV-related fibrosis[515]. Additionally, wild bitter melon extracts can downregulate GPX4 and SLC7A11 in activated HSCs by inducing ferroptosis[516]. Two other proteins regulating ferroptosis in HSCs could be the future targets in the treatment of liver fibrosis: ZFP36/TTP and ELAVL1/HuR. These are critical regulators of HSCs ferroptosis[371,513]; ZFP36 protects against ferroptosis and ELAVL1 contributes to ferroptotic cell death.

Three more diseases may be benefited from ferroptosis inhibitors. Fer-1 improves I/R-mediated liver disease[59,224,276]. ALF is also a candidate for similar treatment based on experimental data. Glycyrrhizin, an active constituent of the licorice root, reduces ferroptosis during ALF, inhibiting oxidative stress through the Nrf2/HO-1/high mobility group box 1 pathway[517]. Finally, reduction of liver iron load will most certainly benefit ALD. Phlebotomy, however, is not recommended in patients with ALD.

An interesting approach to reduce iron load in ALD is the stabilization of erythrocytes and associated reduction in hemolysis. Administration of N-acetylcysteine or protective heme carriers like haptoglobin and hemopexin has been tested. Erythrocyte stabilizers include vitamins such as B12 or folate[281]. Ferrostatin-1 can also reduce alcoholic liver damage[276], indicating participation of ferroptosis in ALD progression. Dimethylfumarate reduces lipid peroxidation and alleviates liver cell ferroptosis leading to ALD improvement in a murine model[275]. Currently, no effective treatment can be recommended for ethanol-induced iron overload. Modulation of ferroptosis for the treatment of chronic liver diseases has been recently reviewed[282].

NUTRIENTS AS TREATMENT OPTIONS OF LIVER IRON OVERLOAD

Vitamin A

Retinoid signaling is decreased in the livers of humans and mice with NAFLD[518,519], and is epigenetically silenced in HCC[520]. Administration of the synthetic retinoid tamibarotene improved oxidative stress and iron deposition in iron-fed mice. Retinoids downregulate the hepatic expression of HJV, leading to liver hepcidin downregulation and ferroportin upregulation[521,522]. Retinoids also attenuate insulin resistance and hepatic steatosis in a murine model of NAFLD[523,524]. Attenuation of hyperinsulinemia may prevent the development of HCC in NAFLD[525].

Vitamin C

A very large observational study with more than 8000 participants demonstrated that dietary vitamin C supplementation decreases plasma ferritin levels[526], indicating that vitamin C limits iron deposition and thereby increases iron mobilization. In a murine model of ALD, vitamin C administration was shown to restore hepatic hepcidin and downregulate intestinal ferroportin, leading to HIO amelioration [527]. Therefore, it is reasonable to supplement vitamin C in ALD and chronic HCV patients with hepatic iron deposition.

Vitamin D

Evidence from patients with thalassemia major and hereditary hemochromatosis indicates that iron overload suppresses vitamin D, as there is a negative correlation between liver iron and 25-hydroxyvitamin D levels[528-530]. In hereditary hemochromatosis, levels of vitamin D are partially restored after phlebotomy[531]. Moreover, vitamin D depletion exacerbates HIO in HJV knockout mice, an effect that is corrected by the administration of the calcium channel blocker verapamil but not by vitamin D supplementation[475,532]. These results indicate a link between iron and calcium and justify the use of calcium channel blockers as a treatment modality for iron deposition in patients with decreased levels of vitamin D, as is frequently observed in ALD, NAFLD, and chronic HCV[533-536].

Zinc

Zinc-deficient diet has been shown to lead to increased plasma ferritin and development of HIO in rats, while zinc supplementation returns liver iron to normal[537]. Clinical studies also indicate that iron deficiency anemia is frequently associated with zinc deficiency[538,539], implying a physiological crosstalk between iron and zinc. For example, zinc plus iron administration in rats has been demonstrated to ameliorate anemia more efficiently than iron alone[540]. The therapeutic effects of zinc on chronic liver diseases have been reviewed[541].

Folate

The Solute Carrier Family 46 Member 1 (SLC46A1) is the major importer of hemeiron in the duodenum, and it is also present in the liver. In murine liver-specific SLC46A1 knockdowns, its role in liver iron deposition has been investigated. In these studies, SLC46A1 was found to take up heme in the liver and contribute to hepatic iron deposition. Interestingly, heme inhibited folate uptake after downregulation of SLC46A1 expression, but folate supplementation had no effect in heme uptake and SLC46A1 expression indicating that folate deficiency was the result of secondary liver heme uptake excess[542]. Accordingly, the combined administration of iron and folate in rats also significantly reduced liver iron compared with iron alone[543].

Riboflavin

Contrary to the agonistic use of the previously discussed nutrients in the treatment of liver iron deposition, riboflavin antagonists, such as galactoflavin, may be used in HIO[544]. This is because riboflavin deficiency leads to a decrease in iron absorption[544,545]. A detailed discussion on the role of nutrients in chronic liver diseases was recently published[546].

CONCLUSION

It is well documented, that iron in the liver is a double-edged sword. It is a necessary element in many metabolic pathways, but it is equally harmful if either the amount or its cellular localization are unbalanced. Increased iron deposition negatively affects most chronic liver diseases. The interplay with lipid metabolism prompted an extensive investigation for the role of iron in NAFLD/NASH. Moreover, the description of ferroptosis as a discrete form of regulated hepatocyte death opened the way for the therapeutic modulation of iron overload in many diseases. Interestingly, most liver diseases are benefited by ferroptosis inhibition. A notable exception is HCC, where the therapeutic target is ferroptosis induction.

The current evidence involves the integration of information from experimental models and less so, from patient findings. Further experimental *in vitro* and *in vivo* investigations are warranted to find more suitable molecules with wider availability and better specificity that could regulate ferroptosis. In this context, it is interesting to note that many natural products may influence iron metabolism and ferroptosis. Furthermore, it should be stressed that clinical trials involving ferroptosis regulation are scarce and sometimes inconclusive. Therefore, to draw valid conclusions, further well-designed randomized trials in humans are urgently required.

FOOTNOTES

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MINIREVIEWS

COVID-19 and the liver: Are footprints still there?

Tarana Gupta, Hemant Sharma

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Abstract

The coronavirus disease 2019 (COVID-19) hit the entire world as a global pandemic and soon became the most important concern for all patients with chronic diseases. An early trend in higher mortality in patients with acute respiratory distress attracted all researchers to closely monitor patients for the involvement of other systems. It soon became apparent that patients with chronic liver diseases are at increased risk of mortality given their cirrhosis-associated immune dysfunction. Additionally, liver function abnormalities were noted in patients with severe COVID-19. Profound cytokine storm, direct viral infection, drugs and reactivation of viral infections were causes of deranged liver functions. Here, we discuss the relation between COVID-19 and chronic liver disease, specifically cirrhosis, hepatitis B, hepatitis C, and non-alcoholic fatty liver disease (NAFLD), as well as the liver manifestations of COVID-19. The metabolic syndrome, obesity, diabetes mellitus and NAFLD were found to worsen outcome in different studies reported worldwide. Decompensated cirrhosis should be considered a risk factor for death and severe COVID-19. Recently, COVID-19 related cholangiopathy has also been reported with changes of secondary sclerosing cholangitis. The long-term persistence of viral antigens in gut epithelia raises concern regarding the future risk of autoimmune liver diseases.

Key Words: COVID-19; Chronic liver disease; Cirrhosis; Liver injury; Transaminases; Non-alcoholic fatty liver disease; Post-acute COVID-19 syndrome

Core Tip: Coronavirus disease 2019 (COVID-19) and liver involvement have been a major concern since the beginning of the COVID-19 pandemic. Deranged liver functions with raised transaminases were reported in patients with severe COVID-19. On the other hand, acute hepatitis or liver failure was uncommon. Severe acute respiratory syndrome coronavirus 2 virus associated cytokine surge, systemic inflammation, direct viral infection, drugs such as remdesivir, steroids, and lopinavir-ritonavir were the main causative factor in raised transaminases. Patients with pre-existing chronic liver diseases especially non-alcoholic fatty liver disease were found to be risk factors for increased mortality in patients with severe COVID-19.

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INTRODUCTION

In December 2019, severe acute respiratory syndrome (SARS) caused by SARS- coronavirus 2 (SARS-CoV2), which belongs to the Coronaviridae family, was first detected in Wuhan, China. It soon spread to the rest of the world, and was declared a global pandemic in March 2020. In mild cases, the symptoms included fever, cough, body aches, malaise, loss of taste and smell. Approximately 15% of patients would eventually have respiratory compromise, hypoxia, and the need for invasive mechanical ventilation. Finally, multi-organ failure, coagulopathy, disseminated intravascular coagulation, acute respiratory distress syndrome, and hypoxia would follow. Over a period of more than 2 years, multiple waves of coronavirus disease 2019 (COVID-19) were observed in different geographical regions. As the virus mutated, there were many shifts in the clinical presentation. New symptoms of predominantly the upper respiratory tract such as sneezing and rhinitis, gastrointestinal symptoms such as diarrhea and non-specific abdominal pain, cardiac symptoms such as arrhythmias, ocular and neurological symptoms were reported. Additionally, as patients underwent more investigations, dysregulated coagulation and thrombosis were documented. Overall, liver involvement such as elevated liver enzymes ranged from 14% to 53% of patients in various studies. However, acute hepatitis or liver failure was uncommon. Furthermore, different studies worldwide have shown that non-alcoholic fatty liver disease (NAFLD), diabetes, hypertension, and obesity are significant risk factors for severe COVID-19.

A lockdown and a ban on air travel during the COVID-19 pandemic helped keep people with chronic illnesses at home. Their overall exposure to COVID-19 and other pathogens was constrained. Mild COVID-19 patients were isolated and quarantined in accordance with protocol and did not frequently undergo examinations. Patients with serious illnesses, however, were the only ones who underwent in-depth examinations. As a result, the majority of research mainly included individuals with serious diseases.

METHODS

We searched PubMed, Google Scholar, and Google from January 2020 to August 2022, for articles written in English that describe the liver effects of COVID-19, using the search terms "coronaviruses and liver", "COVID-19 and liver", "COVID-19 and liver symptoms", "COVID-19 and hepatic", "COVID-19 and liver function test", "COVID-19 and liver inflammation", "SARS-CoV-2 and liver", "COVID-19 and NAFLD", "COVID-19 and non-alcoholic fatty liver disease", "COVID-19 and non-alcoholic fatty liver disease", "COVID-19 and hepatitis", and "COVID-19 and Vaccine". Reference lists of the articles were scanned to identify any additional studies. The title and abstract of each article were read for the initial selection and then the full-text articles were read on availability. Reference lists of the full-text articles were scanned to identify any additional studies. All types of research articles, including original research articles, reviews, case series, short communications, and case reports were considered. Of the 667 articles identified, 313 were studied for this review.

SARS-COV2 HEPATOTROPISM

Due to a lack of significant laboratory testing and tissue biopsies from patients who were actively infected with SARS-CoV2, the mechanism of its replication is still not entirely understood.

SARS-CoV2 is an enveloped positive sense single stranded RNA virus with almost 80% identity with SARS-CoV. It has 4 structural proteins namely nucleocapsid, spike (S), membrane and enveloped proteins. The spike protein has multiple protrusions from the cell surface giving the virus its appearance and name. The angiotensin-converting enzyme 2 (ACE) receptors are the potential site of entry for SARS-CoV2. ACE2 receptors are abundantly present on alveolar epithelium, lung, nasal epithelium *etc.* They are also present in fewer numbers in intestinal epithelium and liver[1]. The spike protein having two subunits S1 and S2 interacts with the ACE2 receptor for virus entry. However, ACE2 receptors are not sufficient alone and transmembrane serine proteases 2 (TMPRSS2) in addition to basic amino acid cleaving enzymes (FURIN) are essential for virus entry. According to single cell RNA sequencing analysis, hepatocytes have less co-expression of TMPRSS2 and ACE2 receptors. In the liver, cholangiocytes and sinusoidal endothelial cells have the highest expression of the ACE2 gene in almost 60% of the cell population as compared to hepatocytes (3% cells)[2,3]. Thus, a tissue or organoid model is required to understand the permissibility of liver cell types to SARS CoV-2 infection. Zhao *et al*[4] created human liver ductal organoids that were able to replicate SARS-CoV2 infection and expressed ACE2 and TMPRSS2. This suggests that the bile duct epithelium may be able to support pseudoparticle invasion. Despite a higher number of SARS-CoV2 virus receptors and a higher risk of infection of bile duct epithelia, COVID-19 does not follow a cholestatic pattern[5].

Studies conducted before the COVID-19 pandemic indicated that patients with hepatitis C virus (HCV)-related cirrhosis had 30 times higher ACE2 receptor expression on hepatocytes than healthy individuals[6]. The overexpression of ACE2 and TMPRSS2 has also been documented in obesity and nonalcoholic steatohepatitis patients, but not in patients with steatosis alone[7]. ACE2 is an interferon-inducible gene found in human respiratory epithelia, possibly SARS-CoV2 hepatotropism can be potentiated by the effects of systemic inflammation on hepatocytes and can lead to hepatocyte injury[8,9].

Additionally, ACE2 receptors are found in intestinal epithelia/enterocytes and SARS-CoV2 RNA has been documented by polymerase chain reaction in stool up to one week after recovery from respiratory illness. The latest data suggests that viral protein and RNA are found in intestinal biopsies for several months after resolution of respiratory illness[5,10].

In a study from Italy, postmortem wedged liver biopsy samples from 48 patients dying from severe COVID-19 were examined[11]. The results revealed vascular abnormalities such as sinusoidal and partial to complete portal venous microthromboses in almost 100% of samples. Additionally, mild portal inflammation, portal fibrosis, microvesicular and macrovesicular steatosis were documented in 66%, 60%, and 50% of patients, respectively. The latter finding is probably related to pre-existing liver disease such as NAFLD, as suggested by the presence of metabolic risk factors which were more prevalent in this patient group. Electron microscopy of these biopsies also revealed potential coronavirus-like particles, mitochondrial edema, and apoptosis of hepatocytes. However, comprehensive proteomic analysis of autopsy tissue from 19 patients with COVID-19 did not find signs of viral replication[12].

Furthermore, proteomic profiling revealed disrupted oxidative phosphorylation, fatty acid oxidation, and up-regulated immunological activators and profibrotic pathways. It is possible that hepatic steatosis, coagulative necrosis, and multi-organ dysfunction were all linked to mitochondrial dysfunction, dysregulated oxidative phosphorylation, *etc*[13].

LIVER FUNCTION TEST AND COVID-19

Despite higher SARS-CoV2 receptor expression on cholangiocytes and SECs, liver function derangement is usually in the form of a mild elevation in liver enzymes [1-2 upper limit of normal (ULN)][14-16].

Singh *et al*[17] showed that the presence of pre-existing liver illness has no effect on the incidence of liver enzyme elevations, although patients with pre-existing liver disease had a higher mortality rate.

In COVID-19, SARS-CoV2 induces a systemic inflammatory response and the release of cytokines. The predominant molecules are interleukin-6 and tumor necrosis factor alpha (TNF-alpha). Elevated cytokines result in hepatocyte inflammation and injury with liver ischemia, hypoxia, worsening of already existing chronic liver disease (CLD) and/or toxicity of medications used to treat the illness (Figure 1). Hepatic congestion as well as potential direct infection of hepatocytes although uncommon may also result in the release of transaminases[18].

However, indicators of muscle breakdown or systemic inflammation did not correlate with serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in hospitalised COVID-19 patients[19,20].

As AST level was frequently observed to surpass ALT level throughout the course of COVID-19, this was similar to patients with alcoholic liver disease, ischemic hepatitis and cirrhosis compared to a traditional hepatocellular pattern where ALT level is greater than AST[19]. Possibly, COVID-19 related mitochondrial dysfunction results in hepatic steatosis and altered hepatic perfusion is the result of sinusoidal microthrombosis[11,21-23].

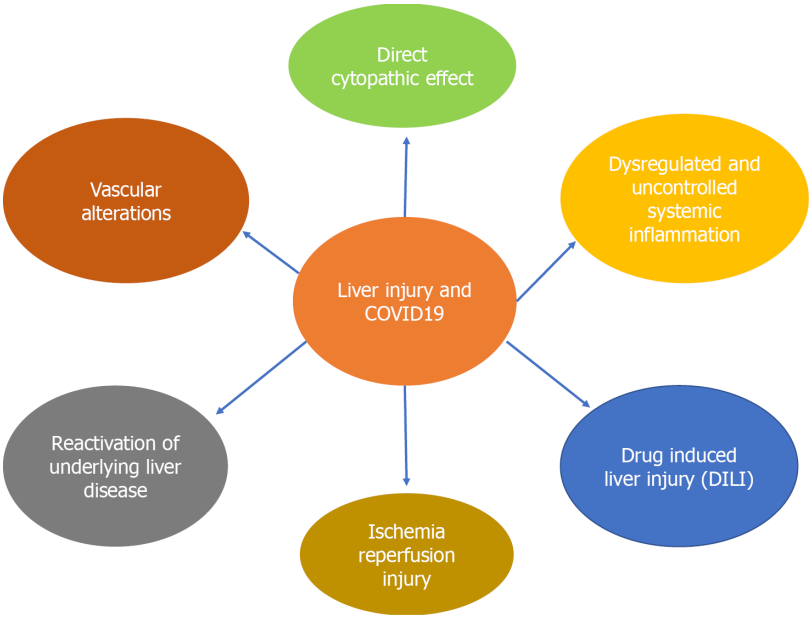

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Figure 1 Effects of COVID-19 and liver injury interaction. COVID-19: Coronavirus disease 2019; DILI: Drug induced liver injury.

Respiratory epithelia involvement by SARS-CoV2 leads to defective oxygenation and the release of cytokines causes peripheral vasodilation and reduced tissue perfusion; the resultant perfusion and oxygenation defect causes systemic hypoxia which is a contributory factor in hepatocyte injury[24].

Early in the pandemic as no definitive treatment was available, experimental therapies in the form of drugs such as tocilizumab, remdesivir and lopinavir-ritonavir were used, which are known to cause hepatic injury[25-29]. Remdesivir was documented to cause elevations in liver enzymes in different studies[30,31]. Tocilizumab was well known for its risk of hepatitis B virus (HBV) reactivation and screening of hepatitis B and hepatitis C was advised before its use.

Ponziani *et al*[32] and Yip *et al*[33] showed that elevations in liver enzymes were associated with an increased incidence of shock, ICU admissions and invasive ventilation. However, these studies could be biased as hospitalized patients with severe disease undergo intensive monitoring of liver function (which increases the chances of detecting liver injury) as compared to home isolated patients with mild disease due to quarantine.

Some studies have suggested that there is no apparent correlation between liver function derangement and mortality[34,35]. Others have suggested an increased risk of death in patients with ALT levels > ULN[16,36,37].

According to Bangash *et al*[38], elevated liver transaminases linked to COVID-19 are more likely caused by severity of the disease, in which the host's reaction and iatrogenic factors such as medication and invasive ventilation cause bystander liver injury and thus explain its link to mortality in a manner similar to that of sepsis[38]. Because of this, clinicians must focus more on these factors than just elevated aminotransferases especially in patients with no pre-existing liver disease.

COVID-19 AND CLD

In the early days of the COVID-19 pandemic, the hepatology community worked fast to establish the risk of SARS-CoV2 acquisition and harmful COVID-19 outcome in pre-existing CLD. According to data from major case series and population-level electronic health records during the first global spike, patients with CLD were not overrepresented, indicating that these diseases did not make patients more susceptible to infection[15,39]. In fact, a significant North American study discovered that people with cirrhosis had a decreased probability of SARS-CoV2 positivity, probably due to improved awareness, testing, and patient adherence to public health recommendations for home isolation and quarantine. However, it is now evident that individuals with cirrhosis are more likely to experience negative COVID-19 outcomes after infection, including mortality. Multiple lines of evidence, such as findings from the international registries SECURE-Cirrhosis and COVID-Hep[40], sizable observational cohorts such as the COVID-Cirrhosis-CHESS group[41], and population-level data, have all been used to support this. These registries were created early in the pandemic and interestingly, due to the emergence of the new Delta and Omicron variants as well as the introduction of vaccines, the relation between COVID-19 and liver will continue to evolve.

In a large registry cohort of 729 patients from 29 countries, it was discovered that mortality in individuals with cirrhosis after SARS-CoV2 infection was 32% overall, with case fatality increasing gradually with each Child-Pugh (CP) class (CLD without cirrhosis: 8%, CP-A: 19%, CP-B: 35%, CP-C: 51%)[42]. The rates of invasive mechanical ventilation, renal replacement treatment, and intensive care unit (ICU) hospitalisation all showed similar stepwise trajectories. Additionally, after adjusting for age and comorbidities, patients with decompensated cirrhosis (CP-B and CP-C) had a considerably higher probability of dying than patients without cirrhosis who tested positive for SARS-CoV2. Reports of elevated COVID-19 mortality in cirrhosis have been confirmed in two Asian-only registries[43] and in numerous multicenter cohort studies conducted in various geographic locations[44-46]. Iavarone *et al* [44] observed a 30-d mortality of 30% in Northern Italy during the early stages of the pandemic, which was much greater than a historical cohort of patients with cirrhosis hospitalised with bacterial infection [44]. Decompensated cirrhosis was also reported as an independent risk factor of death in CLD patients across 21 North American institutions[45]. Additionally, individuals with hepatocellular carcinoma (HCC) had a seven-fold higher chance of dying from COVID-19 than cirrhotic patients without HCC, indicating that this population may be particularly vulnerable to the side effects of SARS-CoV2 infection. A retrospective French cohort of > 259000 COVID-19 inpatients, including > 15000 with pre-existing CLD, showed that patients with decompensated cirrhosis had a higher adjusted risk of COVID-19 mortality[47]. This was in contrast to the findings in a nationwide Swedish cohort, which failed to identify a connection between cirrhosis and COVID-19 related mortality[48]. Cirrhosis overall, and decompensated cirrhosis in particular, should be considered a risk factor for death and severe COVID-19.

There are various characteristics related to the clinical course of COVID-19 in cirrhotic individuals. First, up to 46% of patients can present with acute hepatic decompensation, usually with new or worsening ascites and/or hepatic encephalopathy (HE)[42]. This can occur between 20% and 58% of the time even in the absence of the usual COVID-19 respiratory symptoms[42,44]. Patients with CLD present with gastrointestinal symptoms more frequently than matched controls[42]. This is linked to a more severe disease trajectory[45], a phenomenon that is widespread in society[49] and is connected to increased intestinal permeability, electrolyte imbalance, and systemic inflammatory load, and is documented in up to 12% to 50%[42-44,46] of patients with COVID-19 and decompensated cirrhosis. In the context of COVID-19, a number of well-known prognostic scoring models have been used to assess cirrhosis, with the CLIF-C ACLF score and CLIF organ failure scores surpassing Model for End-stage Liver Disease, North American Consortium for the Study of End-stage Liver Disease, and CP scores in the international and Latin American cohorts, respectively[42,50]. Actually, the likelihood of recovery rapidly decreases as organ support requirements increase. For instance, patients with CP-C cirrhosis have a mere 21% probability of surviving if admitted to the ICU, and decreases to 10% if mechanical breathing is necessary[42].

Despite the fact that SARS-CoV2 infection causes immediate hepatic decompensation, respiratory failure (71%) and problems related to the liver (19%) are the primary causes of death in individuals with cirrhosis[42]. Hepatic dysfunction and lung damage are likely linked by a number of overlapping pathways (Figure 2), including immunological dysfunction brought on by cirrhosis, coagulopathy, and altered pulmonary dynamics due to ascites and HE[51]. Given that the composition of the gut microbiota has been demonstrated to influence how the host immune system reacts to COVID-19, it is conceivable that intestinal permeability and dysbiosis linked to cirrhosis may also have a negative effect [52,53].

Although COVID-19 in patients with cirrhosis is linked to a significant immediate risk of death, rates of mortality and re-admission at 90 d appear equivalent to patients with cirrhosis alone in those who survive the initial shock[54]. Therefore, it appears that SARS-CoV2 infection does not accelerate the progression of liver disease beyond the course of cirrhosis after the acute infective period. However, up to 4 mo after recovering from acute COVID-19, hepatic MRI alterations, including enhanced T1 signaling, raised fat fraction, and hepatomegaly, have been found in 10% to 28% of otherwise healthy people[55,56]. In both patients with and without underlying CLD, it is unknown what these radiological characteristics following COVID-19 mean clinically long-term. Furthermore, although this remains unexplored and is not considered in the current investigations, these hepatic abnormalities might not be exclusive to COVID-19 and might also be present in individuals recovering from other severe systemic insults.

It is crucial to note that studies undertaken in the years before COVID-19 vaccination and the appearance of viral variants like Delta and Omicron are largely responsible for our knowledge of the disease course in individuals with COVID-19 and cirrhosis. CLD can affect 1% to 11% of people with SARS-CoV2 infection[57]. Numerous liver cirrhosis patients have been shown to have drunk alcohol in an ineffective effort to ward off coronavirus infection, raising the risk of alcoholic hepatitis[58].

Implications of COVID-19 include increased mortality associated with severe COVID-19, increased risk of hepatic decompensation, and decreased routine and HCC surveillance.

Although the acute mortality associated with COVID-19 in patients with cirrhosis is substantial, the rates of death and readmission at 90 d are equivalent to those in patients with cirrhosis alone in those who survived the initial insult[54]. Therefore, SARS-CoV2 infection does not appear to accelerate the course of liver disease beyond the typical history of cirrhosis after the acute infective period.

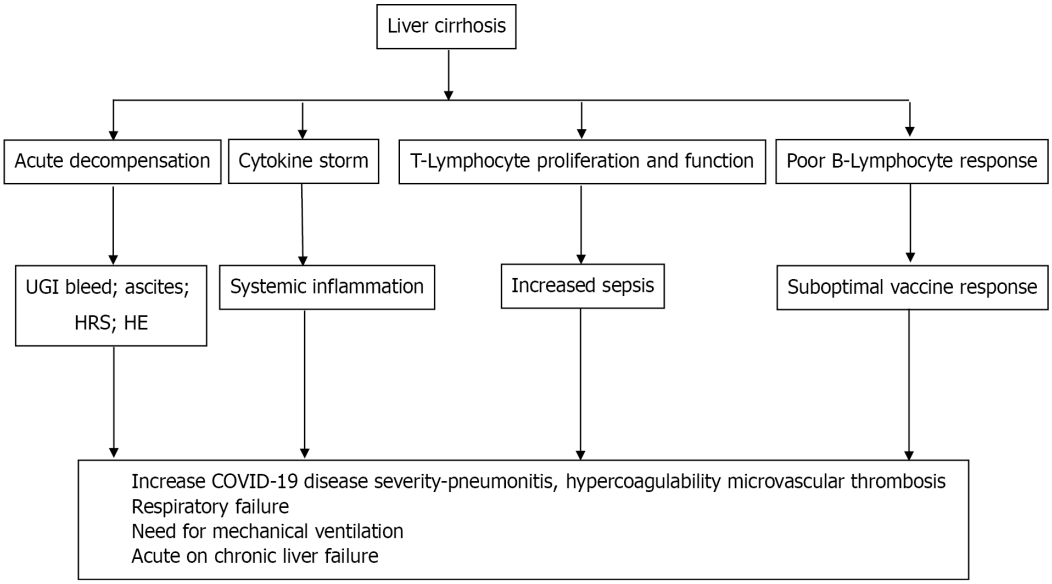

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Figure 2 Course of liver cirrhosis during COVID-19. UGI: Upper gastrointestinal; HRS: Hepatorenal syndrome; HE: Hepatic encephalopathy.

It is well known that infections put people at risk of decompensation (worsening ascites, encephalopathy, or acute kidney injury), and in the case of COVID-19, which is characterized by significant cytokine activation, cytokine-induced hepatocyte apoptosis and necrosis in the presence of decreased liver reserve may result in hepatic decompensation. To rule out COVID-19 as a possible cause, patients with cirrhosis who exhibit decompensation should be evaluated.

CLINICAL OUTCOMES OF PATIENTS WITH INDIVIDUAL UNDERLYING LIVER DISEASES

COVID-19 and Chronic HBV and HCV infection

As there are many etiologies (part of a systemic illness, immune mediated, direct SARS-CoV-2 infection, viral hepatitis, drug-induced, and ischemic hepatic injury) which can cause derangement of liver function tests, one of which is chronic HBV and HCV infection, it is always important to identify these underlying infections[59,60].

Prednisolone and tocilizumab have been used in the treatment of COVID-19, which are known to increase the likelihood of HBV reactivation and flare-up alongside HCV flare-up. When starting COVID-19-related therapy in those with advanced liver disease brought on by HBV and HCV, care must be taken[59,60]. Although the risk/benefit of an intervention is likely to weigh strongly when dealing with COVID-19, established criteria in such cases need to be followed to limit the risk of hepatic decompensation (Table 1).

COVID-19 and NAFLD

Risk factors in the general population for COVID-19 morbidity and mortality include advancing age, obesity, and diabetes[6]. With regard to how NAFLD affects the course of COVID-19, significant differences have been found in various studies. These differences may be attributable to problems in distinguishing the impact of NAFLD from other metabolic comorbidities due to the confounding effect of viral-induced steatosis or due to different diagnostic criteria. The latter point is especially crucial as the hepatology community at large struggles with the proposed classification modifications from NAFLD to metabolic dysfunction-associated liver disease[39]. Studies have shown that obesity is associated with increased severity and mortality in COVID-19. On the other hand, obese patients have a higher prevalence of diabetes, NAFLD, dyslipidemia, hypertension and metabolic syndrome. In a retrospective series of 202 patients with SARS-CoV2 infection, NAFLD was identified as a risk factor for progressive COVID-19, abnormal liver enzyme levels, and extended viral shedding times[61]. A study of 327 participants revealed an association between NAFLD and the likelihood of severe COVID-19 in people under 60 years of age[62]. Similar to this, MRI results from 287 SARS-CoV2 patients (79 positive, 208 negative) showed that obese patients with a concurrent liver fat fraction of less than 10% were three times more likely to develop symptoms of laboratory-confirmed COVID-19 (available as a non-peer-reviewed Preprint only)[63].

Table 1 Studies showing the effect of various etiologies of liver disease on COVID-19

Ref.	Country	Study design	Study population	Sample size	Outcome
HBV					
Anugwom *et al* [70], 2021	China	Letter	Peer reviewed articles with confirmed COVID-19 and HBV information	2054; HBV (*n* = 28)	Inverse relation of HBV with COVID-19
Kang *et al*[71], 2021	Korea	Retrospective, nationwide case-control study	Korean National Health Insurance Service COVID database	7723; HBV (*n* = 267)	Underlying chronic hepatitis B with COVID-19 severity adjusted odds ratio 0.65; 95%CI: 0.57-0.74)
HCV					
Richardson *et al*[15], 2020	United States	Case series	With confirmed COVID-19 and information on HCV infection	5700	HCV infections in < 0.1% (*n* = 3) of COVID-19 patients
Ronderos *et al* [72], 2021	United States	Retrospective single-center	With confirmed COVID-19 and information on HCV infection	1193; HCV (*n* = 50)	HCV infection predictor of in hospital mortality
NAFLD					
Ji *et al*[62], 2020	China	Retrospective	With confirmed COVID-19 and information on NAFLD status	202; NAFLD (*n* = 76)	HSI with disease progression (OR 6.4; 95%CI: 1.5-31.2)
Targher *et al* [64], 2020	China	Prospective observational	Laboratory confirmed COVID-19	310; NAFLD (*n* = 94)	FIB-4 (adjusted OR 1.90, 95%CI: 1.33 to 2.72) or NFS (adjusted OR 2.57, 95%CI: 1.73 to 3.82) with COVID-19 severity
Lopez-Mendez *et al*[65], 2021	Mexico	Retrospective	Medical records of hospitalized COVID-19	155; liver fibrosis (*n* = 69)	FIB-4 with risk of ICU admission (OR 1.74, 95%CI: 1.74-2.68; *P* = 0.023); mortality (OR 6.45, 95%CI: 2.01-20.83, *P* = 0.002)
Sachdeva *et al* [73], 2020	India	Systemic review	-	8142; NAFLD (*n* = 833)	Pooled adjusted 2.358 (95%CI: 1.902-2.923) with severity of COVID-19
Mahamid *et al* [74], 2021	Israel	Retrospective case-control	Medical records of COVID-19	71; NAFLD (*n* = 22)	OR 3.57 (95%CI: 1.22-14.48) with severity of disease
Hashemi *et al* [75], 2020	United States	Multicentre retrospective	Laboratory confirmed COVID-19	363; NAFLD (*n* = 55)	aOR 2.30 (95%CI: 1.27-4.17) with ICU admission
Yao *et al*[76], 2021	China	Retrospective	Laboratory confirmed COVID-19	86; NAFLD (*n* = 38)	OR 11.057 (95%CI: 1.193-102.439, *P* = 0.034) with severe COVID-19
Li *et al*[77], 2022	China and United States	Observational; 2-sample Mendelian randomization	Laboratory confirmed COVID-19	8267; NAFLD (*n* = 136)	OR 0.97 (95%CI: 0.88-1.08, *P* = 0.61) with COVID-19
BCS					
Espinoza *et al* [78], 2021	Brazil	Case report	Laboratory confirmed COVID-19	-	Thrombosis of an abdominal vessel should be considered as a differential diagnosis in patients with undefined abdominal pain and elevated liver biochemical tests
Sh Hassan *et al* [79], 2021	Saudi Arabia	Case report	Laboratory confirmed COVID-19	-	Thromboembolic events could be the first manifestation of COVID-19

COVID-19: Coronavirus disease 2019; HBV: Hepatitis B virus; HCV: Hepatitis C virus; BCS: Budd-Chiari syndrome; HSI: Hepatic steatosis index; FIB-4: Fibrosis-4; NAFLD: Non-alcoholic fatty liver disease; NFS: Non-alcoholic fatty liver disease fibrosis score; 95%CI: 95% confidence interval; OR: Odds ratio.

The chronic low-grade inflammation in NAFLD shifts macrophages from M2 to M1 phenotype and causes activation of hepatic stellate cells and the innate immune system which in collaboration with profound systemic inflammation in COVID-19 leads to hepatocyte injury, necrosis, and apoptosis (Figure 3).

Targher *et al*[64] reported high fibrosis-4 and NAFLD fibrosis scores with increased COVID-19 severity. Similarly, Lopez-Mendez *et al*[65] showed steatosis and fibrosis to be linked to increased ICU admissions. However, due to the constraints of isolation, quarantine and adequate manpower, there was a lack of detailed history and tissue histology; therefore, we do not have comparative studies of liver steatosis, steatohepatitis and fibrosis in relation to COVID-19 severity. The COVID-19 pandemic severely affected hepatology services in terms of early diagnosis, surveillance programs,

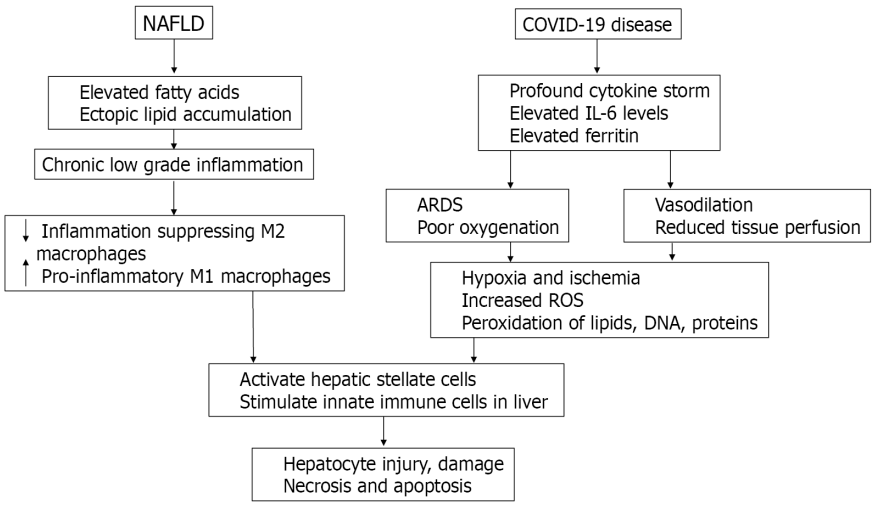

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Figure 3 Complex interplay of non-alcoholic fatty liver disease and COVID-19. COVID-19: Coronavirus disease 2019; NAFLD: Non-alcoholic fatty liver disease; ARDS: Acute respiratory distress syndrome; ROS: Reactive oxygen species; IL-6: Interleukin-6.

implementation of hepatitis B and C eradication programs, *etc* (Table 2).

COVID-19 and autoimmune hepatitis

Very little is known regarding the results of COVID-19 in individuals with autoimmune hepatitis (AIH), a rare form of CLD. The study by Marjot *et al*[66] in October 2020, included more than 1700 participants, and aimed to describe the course of COVID-19 and risk of unfavorable outcomes in 70 individuals with AIH. It was shown that despite the potential reporting of individuals with more severe liver disease, AIH does not significantly increase susceptibility to negative outcomes following SARS-CoV2 infection after several comparisons of non-AIH CLD and non-CLD cohorts. In contrast to the use of immunosuppressive agents, for which no adverse effects were found, age and the severity of baseline liver disease continue to be the most significant drivers of outcome in this patient group[45]. This should reassure patients and medical professionals, and support suggestions that immunosuppressive agents should not be frequently changed or stopped during COVID-19.

COVID-19 cholangiopathy

There are few case reports of secondary sclerosing cholangitis in patients with severe COVID-19 and histologic changes due to cholangiocyte injury and cholangiopathy. These patients had a protracted course and significant liver-related morbidity. Essentially, this condition was noted after recovery of COVID-19; therefore, it was called post COVID-19 cholangiopathy[67].

COVID-19 VIRAL ANTIGEN PERSISTENCE IN THE GUT

Recently, long-term sequelae of COVID-19 have been identified with symptoms of fatigue, insomnia, body ache and cognitive dysfunction. Persistence of viral antigens in gut epithelia have been documented[68]. It is possible that these persistent antigens cause immune dysfunction and low-grade persistent inflammation which manifests in various ways. It could be a basis for immune perturbation in post COVID-19. Its effect on liver in the post COVID era will be an area for research.

ADVERSE EFFECT OF mRNA VACCINES

The effects of mRNA vaccines for COVID-19 prevention have been implicated in the causation of "immune mediated hepatitis" due to the production of antibodies against the spike protein of SARS-CoV2 virus[69]. It will be interesting in the near future to detect autoimmune hepatitis or immune mediated hepatitis prevalence in the community.

Table 2 Impact of COVID-19 pandemic on hepatology services

Decrease	Increase
OPD follow-up and care	Inhospital admission
HBV treatment	Alcohol intake
HCV community level programs	HCC incidence
HCC surveillance and screening	Acute on chronic liver failure
UGI endoscopy	Gastrointestinal bleeding especially variceal bleeding
Liver transplantation	Unhealthy lifestyle
	NAFLD/MAFLD

OPD: Outpatient; COVID-19: Coronavirus disease 2019; HCC: Hepatocellular carcinoma; UGI: Upper gastrointestinal; NAFLD: Non-alcoholic fatty liver disease; MAFLD: Metabolic associated fatty liver disease.

CONCLUSION

During COVID-19, liver enzymes may be mildly elevated and generally recover without treatment. The presence of NAFLD has been linked to increased COVID-19 severity and ICU admissions. Different studies have shown the variable impact of NAFLD on COVID-19 related mortality. In patients with chronic hepatitis B and hepatitis C, a mild COVID-19 course is well tolerated, whereas in moderate-severe COVID-19 requiring steroids and/or tocilizumab, the risk of viral flare and worsening of liver disease is present. Patients with compensated cirrhosis are at increased risk of decompensation after COVID-19. In decompensated cirrhosis, the trajectory of COVID-19 severity and mortality rises with worsening Child-Pugh scores. With emerging evidence of persistent gut viral antigens capable of stimulating the immune system, we should be vigilant for postacute COVID-19 syndrome.

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MINIREVIEWS

Nanomedicine-based multimodal therapies: Recent progress and perspectives in colon cancer

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Abstract

Colon cancer has attracted much attention due to its annually increasing incidence. Conventional chemotherapeutic drugs are unsatisfactory in clinical application because of their lack of targeting and severe toxic side effects. In the past decade, nanomedicines with multimodal therapeutic strategies have shown potential for colon cancer because of their enhanced permeability and retention, high accumulation at tumor sites, co-loading with different drugs, and combination of various therapies. This review summarizes the advances in research on various nanomedicine-based therapeutic strategies including chemotherapy, radiotherapy, phototherapy (photothermal therapy and photodynamic therapy), chemodynamic therapy, gas therapy, and immunotherapy. Additionally, the therapeutic mechanisms, limitations, improvements, and future of the above therapies are discussed.

Key Words: Colon cancer; Nanomedicine; Drug permeability; Drug retention; Multimodal therapies; Therapeutic mechanism

Core Tip: Nanomedicine has exhibited great potential in the colon cancer therapy over the past decades. In this review, we summarize the advances in research on various nanomedicine-based therapeutic strategies including chemotherapy, radiotherapy, phototherapy (photothermal therapy and photodynamic therapy), chemodynamic therapy, gas therapy, and immunotherapy. Additionally, the therapeutic mechanism, limitations, and improvement in these therapies are also introduced. The challenges and future prospect of the nanomedicine-based multimodal therapies for colon cancer are discussed.

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INTRODUCTION

Colon cancer is one of the most intractable gastrointestinal diseases with increasing incidence worldwide[1,2]. For the past few years, human lifestyles and diets have changed markedly with the rapid development of the global economy, which further increases the risk of colon cancer. According to the global cancer statistics, the incidence and mortality of colon cancer were 6.1% and 5.8% in 2018[3], which ranked fourth and fifth among all cancers, respectively. The characteristics of colon cancer are mainly reflected in rapid energy metabolism and proliferation that enhance tumor invasion and metastasis. Therefore, colon cancer has become one of the major unresolved problems in medicine[4,5]. Conventional small molecule chemotherapeutic drugs (such as paclitaxel, doxorubicin, and camptothecin) are unsatisfactory because of their lack of targeting and solubility, and severe toxic side effects. Thus, there is an urgent need to develop novel and efficient therapeutic strategies for colon cancer. In the past decade, the emergence of nanomedicine has shown potential in cancer therapy. Compared with traditional chemotherapeutic drugs, nanomedicine has better tumor targeting because the vascular gaps in tumor tissue are wider than those of normal tissue, so that nanomedicine can penetrate tumor tissue through these vascular gaps but not into normal tissue. Because of the lack of lymphatic reflux in the tumor region, nanomedicine can remain in the tumor tissue, and this mechanism of nanomedicine-based tumor targeting is called the enhanced permeability and retention (EPR) effect [6]. Additionally, various nanoscale drug delivery systems can load the chemotherapeutic drugs to enhance their solubility, which improves their utilization. Finally, nanomedicine is able to combine multimodal therapies to enhance the antitumor effect. Above all, nanomedicine has shown numerous advantages and potential for multimodal therapy of colon cancer.

In this review, we summarize recent progress of nanomedicine-based multimodal colon cancer therapy. First, we introduce all types of organic and inorganic nanomedicine and explore their drug loading, drug release, and tumor targeting. Moreover, the biosafety of nanomedicine is also discussed. Then, we introduce various therapeutic strategies for colon cancer including chemotherapy, phototherapy [photothermal therapy (PTT) and photodynamic therapy (PDT)], radiotherapy, gas therapy, chemodynamic therapy (CDT), and immunotherapy (Figure 1). The therapeutic mechanisms of these approaches are also discussed. Among them, nano drug delivery systems (NDDSs) are widely used to improve the therapeutic effect due to their characteristics of improving the water solubility of chemotherapy drugs, prolonging the blood circulation time, targeted drug delivery, few side effects, and reversing multi-drug resistance. PDT is a new treatment for colon cancer that uses specific wavelengths of light to excite photosensitizers. In the excited state, the photosensitizers transfer energy or electrons to the surrounding oxygen, thus producing singlet oxygen and killing cancer cells. Radiation therapy can cause DNA strand break of tumor cells under X-ray irradiation, and produce high cytotoxic free radicals to damage colon tumor cells. Compared with other reactive oxygen species (ROS) therapies, CDT has stronger *in situ* catalytic ROS generation, higher tumor specificity, and deeper tissue penetration, and does not require additional stimulation, providing a new idea for the future treatment of colon cancer. Gas therapy can enhance drug release, and when used with chemotherapy and synergistic therapy with other therapies, it can improve therapeutic effects, but its application in colon cancer requires extensive studies. Immunotherapy has been widely used in the treatment of colon cancer. The immunogenicity of tumor cells is activated by means of photothermal and ROS, and immunoadjuvant is used to reduce the immunosuppression in the tumor microenvironment and enhance the immune effect. These strategies provide new insights into the clinical treatment of colon cancer. Finally, the main limitations and challenges in the development of nanomedicine for colon cancer are addressed, and future research directions proposed. It is believed that nanomedicine-based multimodal therapy will play an important role in colon cancer.

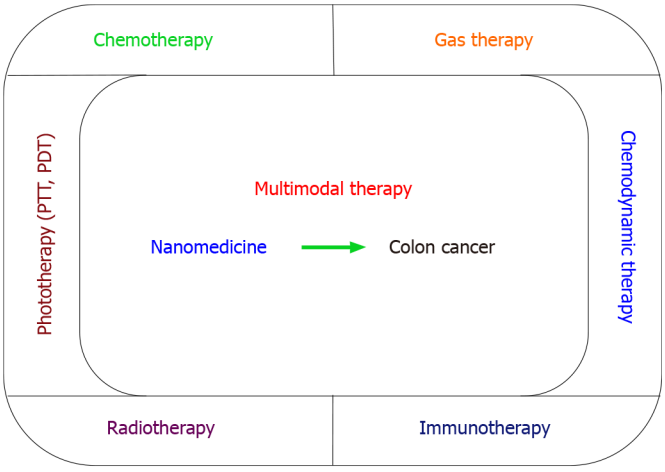

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Figure 1 Schematic illustration of nanomedicine-based multimodal therapies for colon cancer. PTT: Photothermal therapy; PDT: Photodynamic therapy.

MULTIMODAL THERAPIES FOR COLON CANCER

Chemotherapy

Chemotherapy is the core method in current cancer treatment, and various drugs such as 5-fluorouracil (5-FU), platinum drugs, irinotecan, and epirubicin, are widely used[7-11]. However, there are still some problems in conventional chemotherapy: (1) Free small-molecule drugs have a limited half-life *in vivo* and lack of tumor targeting, leading to severe side effect; (2) Poor aqueous solubility of drugs limits their clinical effect; (3) Dense solid tumor tissue hinders drug delivery, resulting in insufficient drug dose in tumor tissue; and (4) Tumor microenvironment, such as hypoxia, low pH, and high H_2O_2 concentration, leads to multidrug resistance. To improve the therapeutic effect of chemotherapy, NDDSs have received extensive attention because of their properties such as improving the aqueous solubility of drugs, prolonging the blood circulation time, achieving targeted delivery to tumors, and few side effects. Various NDDSs have been designed to enhance tumor targeting and aqueous solubility of drugs, leading to improved therapeutic effect[12-15].

Most drugs exhibit poor aqueous solubility and low bioavailability. To solve this problem, Chen *et al*[16] adopted a cucurbituril-based supramolecular chemical strategy to improve the aqueous solubility and long-term circulation of the drugs for enhancing the therapeutic effect of oxaliplatin on colon cancer. Chen *et al*[17] prepared fisetin micelles using monomethyl poly(ethylene glycol)-poly(ε-caprolactone) copolymers. Compared with free fisetin, the micelles exhibited excellent aqueous solubility and cytotoxicity. Additionally, Xiao *et al*[18] used the intermolecular noncovalent interaction of curcumin and irinotecan to self-assemble into nanoparticles, which enhanced the aqueous solubility of curcumin, reduced the side effects of irinotecan, and showed better targeting and therapeutic effect. To prolong the blood circulation of drugs, Jiang *et al*[19] designed OxPt/SN38 nanoparticles to hitchhike on low-density lipoprotein (LDL) particles and accumulate at the tumor site through LDL-receptor-mediated endocytosis, which showed excellent antitumor efficacy in murine tumor models. Liu *et al*[20] developed an active targeting strategy to specifically combine glucose-regulated protein 78 overexpressed on the surface of colon cancer cells with PEGylated WL8 peptide, which enhanced the enrichment of doxorubicin in the tumor region.

Inflammation is an important reason for promoting tumor proliferation, invasion, metastasis, and drug resistance. Therefore, anti-inflammatory drugs such as aspirin and dexamethasone can improve the therapeutic effect of antitumor drugs[21,22]. Natural products such as curcumin and fisetin, which show good anti-inflammatory and antitumor properties, have also been widely used as chemotherapeutic drugs[23-26]. Wang *et al*[27] found that the anti-inflammatory drug dexamethasone significantly enhanced the antitumor activity of carboplatin and gemcitabine and increased their accumulation in tumors, providing a basis for dexamethasone as a chemosensitizer. Ma *et al*[28] developed a pH- and redox-responsive peptide-dexamethasone conjugate (L-SS-DEX) that reduces inflammation and modulates the tumor microenvironment for an effective antitumor effect.

Multidrug resistance is another reason for the failure of chemotherapy. The multidrug-resistance-related proteins such as P-glycoprotein (P-gp) of tumor cells result in significant drug excretion[29,30]. Currently, some NDDSs have been designed to co-deliver P-gp inhibitors or microRNAs to suppress multidrug resistance and enhance the drug sensitivity of tumor cells[31,32]. Sivak *et al*[33] overcame multidrug resistance by simultaneously delivering doxorubicin and the P-gp inhibitor (reversin 121) into cancer cells. The neurokinin-1 receptor antagonists inhibited expression of P-gp to enhance the

chemotherapy effect[34].

Studies have shown that the development of colon cancer is closely related to the gut microbiota, which is involved in regulating the sensitivity of tumor cells to chemotherapy. As a Gram-negative anaerobic bacterium, *Fusobacterium nucleatum* (*F. nucleatum*) is enriched in colon cancer patients, adheres to the intestinal mucosa, and invades epithelial cells to induce carcinogenesis. It can combine with E-cadherin on the surface of colon cancer cells to form a tumor immunosuppressive microenvironment, promote tumor proliferation, and enhance drug resistance of colon cancer cells[35-38]. Therefore, inhibiting the activity of *F. nucleatum* is important for enhancing the efficacy of colon cancer chemotherapy. Lauric acid has a specific inhibitory effect on *F. nucleatum*. Yan *et al*[39] used polyglycidyl ether as a nanodrug carrier, introduced the antibacterial agent lauric acid and oxaliplatin through esterification, selectively inhibited the biological activity of *F. nucleatum*, and improved the resistance of colon cancer cells to oxaliplatin. The antibiotic metronidazole and the chemotherapy drug 5-FU were mixed into the metal polyphenol network coated mesoporous silica nanoparticles (MSNs), and then added with carboxymethyl cellulose to obtain anti-colorectal cancer gel to eliminate *F. nucleatum* in colon cancer and inhibit the drug resistance, and proliferation and metastasis of colon cancer cells[40].

Phototherapy

Phototherapy is an emerging strategy to kill tumor cells by stimulating photosensitizers under light irradiation. In recent years, phototherapy, as a noninvasive treatment, has attracted widespread attention because of its specificity, low toxicity for normal tissues, and excellent antitumor effect. PTT and PDT are two common methods in colon cancer treatment[41-44]. PTT utilizes photosensitizer accumulated in tumor tissue to convert light energy into heat for killing tumor cells under light irradiation (generally near-infrared, NIR), which shows spatiotemporal controllability, high selectivity, and low cost. Recently, NDDSs have been designed to delivery photothermal agents for enhancing tumor targeting. For example, Ren *et al*[45] designed CT26 cell membrane-coated Bi nanoparticles, which had good long-term circulation and tumor homologous targeting ability *in vivo* compared with Bi nanoparticles. In addition, it is reported that epidermal growth factor receptor (EGFR) is abundantly expressed on the surface of some colorectal cancer cells. Shih *et al*[46] combined cetuximab (EGFR inhibitor) with the organic NIR dye IR780 to target colon cancer cells with high EGFR expression for PTT. Excessive H_2S (0.3-3.4 mmol/L) produced by colon cancer cells can promote the proliferation of colon cancer cells and angiogenesis in the tumor area[47,48]. Biocompatible iron oxide nanospindles have been developed, which can efficiently remove endogenous H_2S gas in colon tumor tissues and inhibit tumor growth, and generate FeS *in situ* for magnetic resonance imaging (MRI) and PTT under NIR irradiation[49-51].

PDT is a new method for colon cancer therapy that utilizes light of a specific wavelength to excite a photosensitizer, and the photosensitizer in the excited state transfers energy or electrons to the surrounding oxygen, thereby producing singlet oxygen to kill cancer cells[52]. Various NDDSs have been designed to deliver PDT-based photosensitizers to colon tumors. By adjusting the size of the NDDSs and modifying with hydrophilic groups, the photosensitizers can be passively targeted to the tumor area through the EPR effect. Besides the EPR effect, biomimetic membrane or tumor-specific affinity ligands-modified NDDSs have also been extensively studied for tumor targeting. Xie *et al*[53] designed a translocator protein (TSPO)-targeted photosensitizer (IR700DX-6T) for tumor targeting of photosensitizers *via* combination with overexpressed TSPO in colon cancer cells. Additionally, because of the high expression of EGFR in colon cancer cells, EGFR antibody has been used to target delivery of the photosensitizer IR700, which effectively eradicated colon cancer cells[54]. Traditional photosensitizers have high fluorescence quantum yields in dilute solutions, which leads to weaker fluorescence in the aggregated state. Aggregation of photosensitizers during delivery can lead to reduced ROS yields, so it is crucial to develop novel nanocarriers that efficiently load photosensitizers and prevent their aggregation. Covalent organic frameworks as a class of organic polymers, have attracted much attention because of their excellent biocompatibility and biodegradability. Gan *et al*[55] showed enhanced phototherapeutic effects by adsorbing the NIR dye indocyanine green (ICG) onto the covalent organic framework *via* π–π interaction to prevent its aggregation. In addition to this, aggregation-induced emission luminescence agents have been used to enhance PDT because the agents exhibit enhanced fluorescence emission in the aggregated state[56]. Hypoxia is one of the main reasons for the poor effect of PDT. Thus, researchers have developed a variety of oxygen generators such as hemoglobin, MnO_2, and perfluorocarbon, to increase oxygen in the tumor to enhance the effect of PDT [57-59]. For example, He *et al*[60] designed gold nanocages coated with MnO_2 and hyaluronic acid (HA) for tumor targeting, and MnO_2 was designed to react with the overproduced H_2O_2 in the tumor to relieve tumor hypoxia and enhance the effect of gold nanocage-based PDT.

Radiotherapy

Radiotherapy is a local cancer treatment that is widely applied in clinical therapy. The mechanism of action of radiotherapy is to cause DNA strand breaks in tumor cells and generate highly cytotoxic free radicals under X-ray irradiation to damage tumor cells[61-65]. Radiosensitizers are usually used to boost the effect of radiotherapy against colon cancer[66]. 7-Dehydrocholesterol is utilized as a radiosensitizer,

which can react with ROS to promote lipid peroxidation, double-strand breaks, and mitochondrial damage in cancer cells, enhancing the radiotherapeutic effect[67]. As we know from the mechanism of action of radiotherapy, tumor hypoxia limits the efficacy of radiotherapy; thus, relief of hypoxia by nanomedicine can improve the therapeutic effect. MnO_2 can react with excess H_2O_2 in the tumor to generate oxygen, which can relieve the hypoxic microenvironment, eliminate tumor resistance to radiotherapy, and reshape the immunosuppressive microenvironment. Zhang *et al*[68] designed bovine-serum-albumin-coated MnO_2 as a radiosensitizer. MnO_2 can decompose excess H_2O_2 in the tumor into oxygen to relieve tumor hypoxia and convert tumor-promoting M2 tumor-associated macrophages into antitumor M1-type macrophages to reshape the immunosuppressive microenvironment and eliminate tumor resistance to radiotherapy. In addition, perfluorocarbon is a good oxygen carrier that can be used to delivery oxygen to tumors and reverse hypoxia, leading to enhancement of radiotherapy[69].

CDT

CDT is a promising therapeutic strategy that utilizes endogenously overexpressed H_2O_2 in tumors to generate toxic hydroxyl radicals ($\cdot OH$) through Fenton/Fenton-like reactions catalyzed by metals (Fe^{2+}, Cu^{+}, Mn^{2+}, Mo^{4+}, W^{4+}, Ti^{3+}, *etc.*)[70-73]. Compared with other ROS therapies, CDT has the advantages of stronger *in situ* catalytic ROS generation, tumor specificity, and deep tissue penetration, which does not require additional stimulation. However, the effect of CDT is still limited by its high dependence on tumor endogenous H_2O_2 concentration (10-100 μM) and slow ion release from inorganic nanoparticles [74,75]. The problem of low levels of H_2O_2 in tumor tissue can be solved by directly loading H_2O_2 or encapsulating H_2O_2-producing drugs such as glucose oxidase and calcium peroxide. However, nanocarriers directly encapsulating exogenous H_2O_2 have the risk of leakage causing damage to normal tissues. Therefore, new strategies are urgently needed to address the challenges associated with CDT. Su *et al*[76] used a microfluidic method to prepare a nanogel (DOX@Mn-Alg) composed of alginate (Alg), Mn^{2+}, and doxorubicin as an ideal CDT/chemotherapy synergistic therapeutic nanoplatform, because doxorubicin can activate NADP oxidases to convert oxygen to $\cdot O_2^-$ and then superoxide dismutase further catalyzes $\cdot O_2^-$ to generate endogenous H_2O_2 *via* a disproportionation reaction. Subsequently, the elevated H_2O_2 can be converted into a sufficient amount of $\cdot OH$ through a Mn^{2+}-mediated Fenton-like reaction. Ultimately, DOX@Mn-Alg can rationally combine doxorubicin chemotherapy with Mn^{2+}-mediated CDT and immunotherapy for synergistic cancer treatment. Chen *et al*[77] selected Pd nanoparticles as a CDT reagent, and showed that the ultra-small Pd nanozyme as the core had high catalytic activity and pH selectivity. Under acidic conditions, it exhibited peroxidase activity to produce OH and $^1O_2$, while under neutral conditions, it promoted the decomposition of H_2O_2 to produce O_2 through catalase activity. In terms of biological activity, the bidirectional anisotropic nanocluster not only directly inhibited tumor cells through ROS production, but also induced H_2O_2 production in CT26 cells, which enhanced the therapeutic effect. The nanoparticles inhibited tumor growth in CT26 mice, and improved tumor hypoxia and enhanced the therapeutic effect.

The intracellular glutathione in tumor cells can eliminate the oxidative activity of $\cdot OH$ through powerful reducing activity. Lin *et al*[78] devised a strategy to enhance CDT by inhibiting expression of glutathione in tumors and remodeling the reductive state of the tumor microenvironment, indicating that inhibition of glutathione can improve the effect of CDT. Wang *et al*[79] reported a degradable $MnSiO_3$ nanosystem for CDT/chemical synergistic therapy. First, $MnSiO_3$ nanoparticles were synthesized, and then the surface-initiated living radical polymerization of monomer of SN38 and oligo(ethylene glycol) methacrylate was conducted to obtain the product of CAMNSN@PSN38. Nanoparticles delivered to tumor tissues were gradually biodegraded by glutathione over time, during which SN38 and Mn^{2+} were gradually released. The released SN38 showed a favorable chemotherapeutic effect and increased accumulation of H_2O_2. The interaction of CAMNSN@PSN38 with glutathione depleted glutathione in tumor tissues and led to Mn^{2+} release for CDT and MRI-guided therapy. CAMNSN@PSN38 had a good inhibitory effect on colon tumor growth and assisted MRI-guided imaging through ROS accumulation *in vivo*. Unlike other tumor types, colon tumor shows high expression of H_2S (0.3-3.4 mmol/L), whose reductive activity is stronger than that of glutathione[80,81]. Therefore, in the treatment of colon cancer, the effect of CDT is also limited by endogenous H_2S. Liu *et al* [82] constructed $CuFe_2O_4$ nanoparticles to explore the potential of endogenous H_2S depletion to enhance CDT for colon cancer. $CuFe_2O_4$ nanoparticles remodel endogenous H_2S in colon cancer and enhance the Fenton or Fenton-like reaction of Cu(I) and Fe(II) by a photothermal effect to generate more $\cdot OH$. The results suggest that $CuFe_2O_4$ nanoparticles effectively enhance the effect of CDT by depleting H_2S. In addition, H_2S-responsive therapeutic nanoplatforms have been designed. Xiao *et al*[18] synthesized a copper-based metal-organic framework named HKUST-1 as a smart therapeutic platform. PTT and CDT were activated in the presence of H_2S in colon cancer cells. H_2S-triggered nanosystems can minimize side effects on surrounding normal tissues and precisely inhibit colon cancer growth. Above all, CDT shows potential for colon cancer treatment.

Gas therapy

As an emerging treatment method, gas therapy has attracted research interest in recent years[83-86]. Gas therapy refers to use of H_2S[87], NO[88], CO, *etc.* to kill tumor cells[89]. Liu *et al*[90] designed a nanoplatform (PEG/SCNPs@DMSN-SNO-g-C_3N_4) to release NO under X-ray irradiation, and then NO

reacted with superoxide anions to generate $ONOO^-$ toxic free radicals, leading to apoptosis through mitochondrial damage. NO has been proven to activate innate and adaptive responses of the immune system against tumors. Previous *in vivo* results showed that all NO-treated colon tumor-bearing (CT26 model) mice were resistant to secondary CT26 cell inoculation. Nonsteroidal anti-inflammatory drugs (NSAIDs) are prototypical anticancer agents. NO and H_2S are gaseous mediators with physiological relevance and NSAIDs that possess an H_2S- and NO-releasing moiety have shown beneficial effects. Chattopadhyay *et al*[91] synthesized and characterized a new class of anti-inflammatory NO- and H_2S -releasing compounds. This induced apoptosis, inhibited cell proliferation, and reduced colon tumor growth in a mouse xenograft model. Zhang *et al*[92] designed gas-generating MSNs, which can load ammonium bicarbonate and doxorubicin in the pores, and ICG coated on a polydopamine layer and modified with RGD peptides on the outer surface [M(ABC)-DOX@PDA-ICG-PEG-RGD] for triggering drug release and targeted chemotherapeutic photothermal combination treatment. At high temperature and low pH, the encapsulated ammonium bicarbonate can effectively generate CO_2. The CO_2 can damage the polydopamine layer and accelerate the release of doxorubicin. The results proved the excellent antitumor effect of gas therapy and chemotherapy, as well as good biosafety. Therefore, the gas therapy showed potential for colon cancer therapy.

Immunotherapy

Immunotherapy exhibits potential against colon cancer because it relies on the autoimmune system to attack malignant tumors. Immunotherapy for colon cancer is mainly divided into the following categories: (1) Activation of tumor immunogenicity; (2) Relief of tumor microenvironment immunosuppression; (3) Design of antitumor neoantigen vaccines and novel immune adjuvants; and (4) Design of therapeutic strategies using macrophages as target cells. However, only a subset of cancer patients responds to current immunotherapies because of the low immunogenicity of tumor cells and the immunosuppressive tumor microenvironment. Therefore, new strategies are needed to activate tumor immunogenicity and relieve the immunosuppression of the tumor microenvironment to improve the effect of immunotherapy. Fan *et al*[93] reported pH-responsive core-shell nanoparticles (HCLO NPs) for co-delivery of oxaliplatin intermediate and cytosine-guanine-containing oligodeoxynucleotide (CpG) for colon cancer treatment, and the oxaliplatin intermediate intratumoral injection induced *in situ* antigen production *via* immunogenic cell death. Subsequently, CpG enhanced antigen presentation and promoted production of cytotoxic T lymphocytes (CTLs). The results indicated that the HCLO NPs enhanced the toxicity of oxaliplatin intermediate for CT26 cells and upregulated expression of calreticulin, which exhibited significant immunity and antitumor effect. Hu *et al*[94] integrated HA, pheophorbide A heterodimer, and NLG919 into a supramolecular nanocomposite, which generated ROS under NIR laser irradiation to kill tumor cells, stimulated antitumor immunogenicity, and enhanced intratumoral infiltration of CTLs. The immunosuppressive tumor microenvironment was reversed by NLG919-mediated inhibition of indoleamine 2,3-dioxygenase 1. The results showed that this strategy could effectively kill CT26 colon tumors. Ding *et al*[95] designed liposome-encapsulating phosphatidylinositol 3-kinase γ inhibitor IPI-549 and photosensitizer Ce6 for immunotherapy of colon cancer. When the liposomes were internalized into CT26 cells, ROS were generated under laser irradiation, causing immunogenic tumor cell death. IPI-549 transported by liposomes promoted apoptosis of myeloid-derived suppressor cells and reduced the immunosuppressive activity of $CD8^+$ T cells to inhibit growth of CT26 tumors. Checkpoint inhibitors, such as antibodies that block the programmed cell death protein 1/programmed cell death ligand 1 (PD-1/PD-L1) pathway, are among the most promising immunotherapies for metastatic cancer. However, the responses rates remain low. To solve this problem, Yu *et al*[96] developed nanoparticles with PD-L1 blocking ability, which integrated PTT, antitumor immunity, and PD-1/PD-L1 blockade to enhance antitumor efficacy. In the mouse CT26 bilateral tumor model, intravenously injected nanoparticles accumulated at the tumor site and mediated a strong photothermal effect, eliminated the primary tumor by inducing immunogenic cell death, and elicited strong antitumor immunity. Growth of untreated distant tumors was inhibited by the synergistic effect of systemic antitumor immune activation and PD-L1 blockade. This strategy provided a promising approach for the treatment of metastatic cancer.

The reported immunoadjuvants have many limitations, such as poor cellular uptake and biocompatibility, excessive particle size, single function, and unsatisfactory therapeutic effect. Ding *et al*[97] prepared mesoporous silica-coated upconversion nanoparticles (UCMSs) and used them as a novel immune adjuvant. UCMSs had significant loading of the photosensitizer merocyanine 540, chicken ovalbumin, and tumor cell fragments. The UCMSs exhibited the best synergistic immune enhancement under 980 nm NIR irradiation, with the strongest Th1 and Th2 immune responses, and the highest frequencies of $CD4^+$, $CD8^+$, and effector memory T cells. In addition, nanovaccine UCMSs inhibited tumor growth more effectively and improved survival of tumor-bearing mice compared with PDT or immunotherapy alone, indicating that UCMSs have higher immunotherapeutic efficacy and clinical potential. As a new tumor vaccine based on zymosan shell particles[98], GP-Neoantigen can stimulate the body to generate a strong antigen-specific $CD8^+$ T cell immune response and an immune response to a variety of neoantigen peptides, and thereby be used for effective tumor treatment. The vaccine induced strong specific $CD8^+$ T cell immune responses and humoral immune responses *in vivo*, which also showed strong tumor growth inhibitory activity in the CT26 colon cancer model. Binding to Toll-

like receptor agonists PolyI:C and CpG 2395 enhanced the antitumor effect and achieved complete tumor clearance. These results provide broad possibilities for further clinical promotion and personalized vaccine therapy.

M2 macrophages are polarized by stimulatory factors in the tumor microenvironment and promote tumor growth. They are involved in limiting T cell function, tumor angiogenesis, and tumor invasion and metastasis. Increasing the ratio of M1/M2 macrophages in the tumor microenvironment is a promising cancer immunotherapy strategy. An erythrocyte membrane nanoparticle encapsulating *Porphyromonas gingivalis* can modulate the ratio of M1/M2 macrophages for cancer immunotherapy[99], and such nanoparticles inhibited the growth of primary and secondary tumors of CT26 colon cancer under the action of laser and anti-PD-1. Immunotherapy based on nanomedicine has been widely used in cell and animal models, and has shown good anti-tumor efficacy. It is expected to become one of the most potential therapeutic means in cancer treatment.

CONCLUSION

Several advanced nanomedicine applications have been developed for colon cancer therapy, which overcome the poor tumor targeting and efficacy of conventional drugs. This review presents various organic- and inorganic-based nanomedicines applied in colon cancer therapy using CT26 cells as the tumor model. We have introduced the mechanism of nanomedicine-based therapeutic strategies including chemotherapy, phototherapy (PTT and PDT), radiotherapy, gas therapy, CDT, and immunotherapy. These multimodal therapeutic strategies based on nanomedicine against colon cancer have shown excellent antitumor effect and potential.

Although the nanomedicine-based multimodal therapies have shown a superior effect against colon cancer, several limitations need to be overcome in future development. The first limitation is the unsatisfactory tumor penetration of nanomedicine. Drug delivery *in vivo* includes circulation, accumulation, penetration, internalization, and release. Poor tumor penetration has become a long-standing problem for the development of nanomedicine, which leads to the survival of tumor stem cells in deep tumor sites. The reason is the serious hinders of dense extracellular matrix and elevated tumor interstitial pressure. Thus, there is an urgent need to develop novel strategies to enhance tumor penetration of nanomedicine. The second limitation is obstruction of various therapies by the tumor microenvironment. For example, tumor hypoxia limits oxygen-dependent therapy such as PDT and radiotherapy. Additionally, M2 tumor-associated macrophages construct the tumor immunosuppression environment, which limits the effect of immunotherapy. Not only that, the immune checkpoint protein on the tumor cell inhibits the recognition and combination of cytotoxic T cells. Therefore, reversing the adverse effects of the tumor microenvironment is the key to improving the therapeutic effect of nanomedicine. It is expected that nanomedicine-based multimodal therapeutic strategies will have potential for clinical translation into colon cancer therapy.

FOOTNOTES

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MINIREVIEWS

Gaseous metabolites as therapeutic targets in ulcerative colitis

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Abstract

Diet therapies are currently under-utilised in optimising clinical outcomes for patients with active ulcerative colitis (UC). Furthermore, existing dietary therapies are framed by poorly defined mechanistic targets to warrant its success. There is good evidence to suggest that microbial production of gaseous metabolites, hydrogen sulfide (H_2S) and nitric oxide (NO) are implicated in the development of mucosal inflammation in UC. On a cellular level, exposure of the colonic epithelium to excessive concentrations of these gases are shown to promote functional defects described in UC. Hence, targeting bacterial production of these gases could provide an opportunity to formulate new dietary therapies in UC. Despite the paucity of evidence, there is epidemiological and clinical data to support the concept of reducing mucosal inflammation in UC *via* dietary strategies that reduce H_2S. Several dietary components, namely sulphur-containing amino acids and inorganic sulphur have been shown to be influential in enhancing colonic H_2S production. More recent data suggests increasing the supply of readily fermentable fibre as an effective strategy for H_2S reduction. Conversely, very little is known regarding how diet alters microbial production of NO. Hence, the current evidence suggest that a whole diet approach is needed. Finally, biomarkers for assessing changes in microbial gaseous metabolites in response to dietary interventions are very much required. In conclusion, this review identifies a great need for high quality randomised-controlled trials to demonstrate the efficacy of a sulphide-reducing dietary therapy for patients with active UC.

Key Words: Diet; Ulcerative colitis; Hydrogen sulfide; Nitric oxide; Sulphide-reducing diet

Core Tip: There is room to develop efficacious dietary therapies in ulcerative colitis (UC) by targeting underlying pathogenic mechanisms. Emerging data indicates that dietary factors play a significant role in modulating two gaseous metabolites, hydrogen sulphide and nitric oxide, that affect the integrity of the colonic mucosal barrier in UC. These gases are produced by the colonic microbiota in response to sulphur-containing protein and to a lesser extent, inorganic sulphur (sulphates and sulphites), but suppressed by the presence of fermentable fibre. Preliminary work suggests that a multi-prong diet that targets reduction of these gases have therapeutic potential and further randomised-controlled trials are underway.

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INTRODUCTION

Ulcerative colitis (UC) is characterised by chronic inflammation of the colonic epithelium as a result of an aberrant immune response to poorly understood initiating triggers[1]. Diet is a well-recognised environmental factor in the development of UC[1,2], but remains an under-utilised therapeutic tool amongst physicians and dietitians alike. Dietary management is currently directed at providing supportive symptomatic management. However, in the recent years, there has been a dogma shift towards harvesting dietary therapies with mechanistic targets for the induction of disease remission, as evidenced by the growing number of review articles in the area[3-5].

Whilst most research have been focused on altered immune regulation in the early initiative events, there is now a good body of evidence generated over the last 20 years suggesting that UC is an epithelial disease[6]. Metabolic defects in the colonic epithelium are central in its pathogenesis and may be responsible for mucosal barrier breakdown[7]. In turn, microbial metabolites such as hydrogen sulfide (H_2S) and nitric oxide (NO) that are toxic at excessive concentrations, may further exert injurious effects on the epithelium[8]. Diet is a major factor in colonic production of these metabolites. Hence, dietary strategies that minimise their production mechanistically may have therapeutic benefits in UC. This review aims to examine the evidence for H_2S and NO as causative agents in UC, the influence of diet on their colonic metabolism and to explore the rationale as well as evidence to date for dietary strategies targeting these gaseous metabolites as a therapy in UC.

COLONIC PRODUCTION OF H_2S & NO

Luminal H_2S is derived solely from metabolic activities of the microbiota, namely from fermentation of sulfur-containing amino acids and dissimilatory sulfate reduction[9]. Approximately 6-18 g/d of proteinaceous substrates are delivered to the colon for fermentation, the bulk of this originating from undigested dietary protein and a smaller proportion from endogenous protein secretions[10]. A range of protein-fermenting microbes with the capacity to generate H_2S have been reported including *Escherichia coli, Clostridium spp., Bacteroides spp.* and *Klebsiella pneumonia*[10]. In contrast, the capacity to reduce sulfate within the microbiota appears to be limited. A smaller proportion of malabsorbed dietary inorganic sulfur (0.3-8 mmol/d)[11] reach the colon as substrates for dissimilatory sulfate reduction. Sulfate- and sulfite-reducing bacteria such as *Desulfovibrio spp.* and *Bilophila wadsworthia* are highly specialised microbes with capacity for sulfate reduction[11].

On the other hand, two major sources of luminal NO are known: (1) Mucosal production from arginine; or (2) Anaerobic bacterial denitrification which reduces nitrates to nitrites and to NO[8]. To date, little work has been done to examine microbial populations capable of denitrification. Hence, the understanding of microbial pathways for gaseous production has important implications not only as potential therapeutic targets but has significant relevance for manipulation of dietary substrates.

ROLE AS LUMINAL TOXINS IN PATHOGENESIS OF UC

The most compelling argument for the colonic epithelium as the primary defect in UC has been derived from *ex vivo* studies showing diffuse structural and functional abnormalities in the absence of histological or endoscopic inflammation[12-14]. A key functional defect identified is the impaired uptake and oxidation of butyrate by colonocytes for energy[15,16]. As a result, the energy-starved colonic epithelium has limited ability to perform other metabolic functions including the maintenance of

barrier function. Furthermore, reduced structural integrity of the colonic mucus layer was reported by van der Post *et al*[13]. This was characterised by a marked decrease in core mucus components in both inflamed and non-inflamed biopsy samples, with similar findings reported previously[14]. Hence, the induction of mucosal inflammation may occur as a secondary response to the increased intestinal permeability[6].

Several lines of observations support the involvement of luminal H_2S and NO in perpetuating functional defects of the colonic epithelium. These concepts are summarised in Figure 1. First, Levine *et al*[17] showed that faecal release of H_2S was three-fold higher and more rapid in UC patients (both active and quiescent) compared to controls. Additionally, a greater relative abundance and activity of sulfate-reducing microbes, *Desulfovibrio*, has been documented in faecal or mucosal biopsy samples of patients with UC compared to non-UC controls[18,19]. Gut dysbiosis may be the main pathogenic factor of UC, and the higher dominance of sulphate-reducing microbes may potentially contribute to the dysbiosis hypothesised in the pathogenesis of UC. No data currently exists of potential alterations to the abundance of protein-fermenting microbes in UC. Furthermore, in contrast to a healthy colonic epithelium where H_2S is effectively detoxified, enzymatic detoxification activity of H_2S have been shown to be significantly depressed in UC[20]. Finally, elevated luminal H_2S concentrations are shown to be directly proportional to the severity of disease[16,17], providing an evidence base for a pathogenic link with UC. Likewise, direct assessment of luminal NO using a rectal balloon in patients with active UC demonstrated markedly higher rectal NO levels in these patients compared to those with irritable bowel syndrome and healthy controls[21].

Secondly, reduced carbohydrate fermentative ability, as was recently reported[22], and decreased accessibility to short-chain fatty acids[23] may have lead-on effects on altered sulfur metabolism. Insights gained by assessment of intestinal pH responses to dietary manipulation of fermentable fibres suggest that abnormalities in carbohydrate fermentative ability may be region specific[24]. Reduced butyrate utilisation may increase luminal accumulation of H_2S as its regulatory role on detoxification pathways are affected[25]. On the other hand, fibre deprivation may act synergistically with H_2S to increase breakdown of the mucous layer[26].

Thirdly, at excessive concentrations, continuous exposure of isolated colonocytes to combined H_2S and NO *in vitro* can produce extensive disruption of the epithelial barrier by interfering with cell membrane synthesis[8], impeding butyrate oxidation and subsequently, cellular respiration, producing an energy-deficient state as described earlier. This theory was confirmed by Leung *et al*[27] who induced a histological state that was similar to the pathology of UC in the colon of rats administered with sulfates (carrageenan). Furthermore, excessive H_2S and NO may exert other pathogenic effects, including direct immune effects and these are summarised in Figure 1.

Hence, restricting epithelial exposure to luminal H_2S and NO *via* reduced microbial production may hypothetically improve epithelial function and reduce mucosal inflammation in UC, a novel therapeutic strategy that was proposed two decades ago[28] but has only achieved some progress in the last two years. Progress is hampered by difficulties in accurate measurements of luminal H_2S and NO to provide a biomarker for assessing the efficacy of interventions on these metabolites. These challenges are discussed further in the subsequent sections.

DIET AS PRIMARY STRATEGY FOR COLONIC H_2S & NO MANIPULATION

From discussions above, it can be hypothesised that a key strategy in reducing microbial H_2S and NO production is by reducing substrate availability. Food choice represents a rationale candidate for manipulation as substrate delivery to the colon is strongly influenced by dietary intake. Indeed, several lines of evidence exist supporting the efficacy of dietary manipulation on colonic H_2S production. In contrast, the influence of diet on the extent of bacterial denitrification has been inconsistently shown.

First, acute dietary studies in healthy controls changing from low to a high animal protein diet consistently raised faecal H_2S levels[29,30]. Magee *et al*[29] reported this increase in H_2S levels to be linear with increasing intake of red meat (from 0 to 600 g/d). In another study, a four-day animal-based diet specifically increased a sulfite-reducing species, *Bilophila wadsworthia*, while a plant-based diet reduced this cluster[31]. Similarly, the animal-based diet significantly increased sulfide reductases needed for H_2S production[31]. Another source of inorganic sulfur in the diet occurs naturally in the form of glucosinolates in the Brassica vegetables family. However, a two-week diet high in brassica was associated with a reduction in the abundance of sulphate-reducing bacteria in a randomized crossover study with ten healthy adults[32], which seems to indicate that natural inorganic sulfur is not a determining factor in H_2S production associated with sulfate-reducing bacteria. Thirdly, whilst assessment of sulfate-reducing bacteria may be useful, it does not provide a comprehensive picture of functional alterations in microbial H_2S metabolism *in vivo*. Preliminary insights were gained with the use of a gas-sensing technology incorporating real-time, accurate measurements of H_2S to enable further understanding of the extent of dietary influence on microbial H_2S production[33]. A comparison between faecal slurries spiked with cysteine, a sulfur-containing amino acid, and sodium sulfate showed marked differences in faecal H_2S generation, with cysteine vigorously stimulating H_2S over

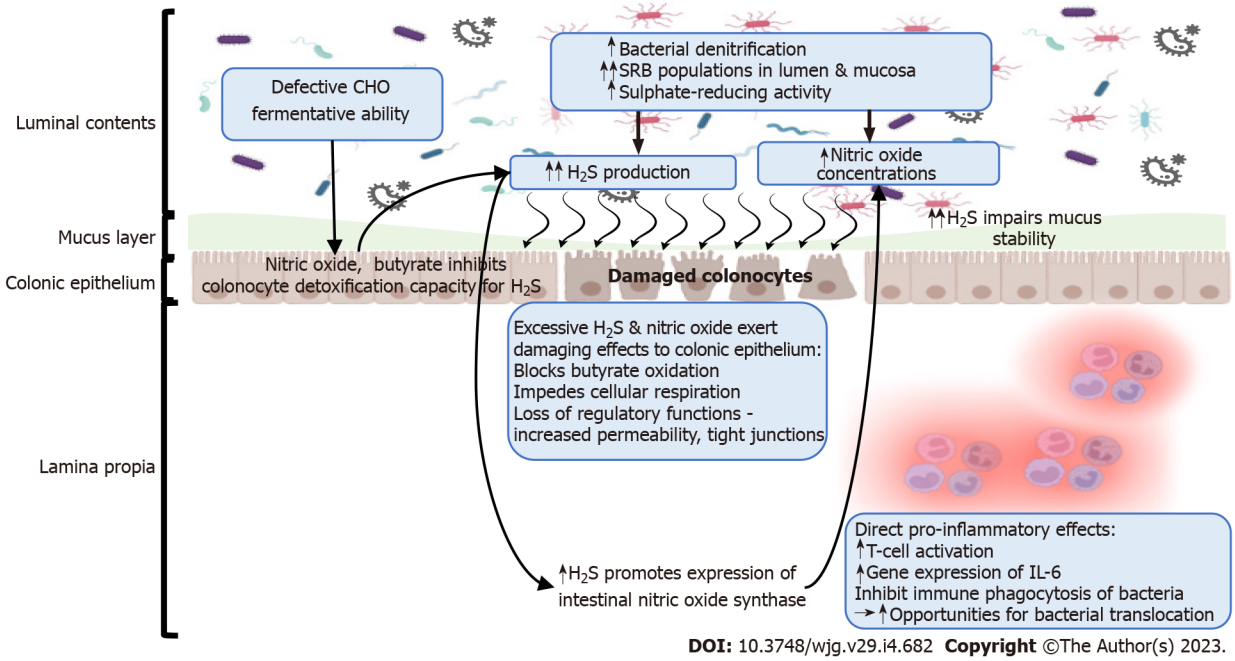

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Figure 1 Proposed mechanisms of microbial metabolites, hydrogen sulfide and nitric oxide, in the pathogenesis of ulcerative colitis *via* a dysfunctional colonic epithelium and breakdown in mucosal barrier function. Figure summarised from references[8,55-57]. CHO: Carbohydrate; H_2S: Hydrogen sulfide; SRB: Sulfate-reducing bacteria; H_2S: Hydrogen sulfide; IL: Interleukin.

sulfate. This finding indicates that protein fermentation may be a major pathway for H_2S production than dissimilatory sulfate reduction. Furthermore, faecal H_2S was effectively reduced by readily fermentable fibres, resistant starch and fructo-oligosaccharides, both of which are prebiotics, and even in the presence of excessive faecal H_2S production using cysteine[33]. The likely mechanism for H_2S suppression by fermentable fibre in the presence of cysteine is the shift from protein to carbohydrate fermentation as microbes preferentially ferment fibre than protein[10]. Suppression of H_2S has been reported by another study where a 1.5-fold increase in total dietary fibre that accompanied the reduction in animal protein had a negative impact on H_2S production[30] and in a second study, the addition of resistant starch to a high meat diet reduced markers of protein fermentation including H_2S [34]. Both inulin and fructo-oligosaccharides, well-established prebiotics were also shown to reduce H2S levels in pigs[35].

In addition, one of the strategies targeting the microbiota is probiotics with specific probiotic strains shown to be effective in inducing remission in active UC[36]. However, its properties on the gut microbiota warrants further investigation, particularly with regards to the influence of different probiotic strains on H_2S production. On the other hand, a promising probiotic treatment for UC is recent development of a 'smart probiotic' where E. coli Nissle 1917 was genetically engineered to detect colonic NO and would theoretically be able to release biologic therapy at the site of elevated colonic NO[37]. This engineered probiotic had previously been shown to have positive impact on the intestinal barrier function, and were able to reduce inflammation in dextran sulfate sodium -induced colitis mice model [38]. Prebiotics are another key player in microbiome manipulation that have been suggested to have a positive effect on the microbiome. Their mechanisms in modulating microbial H_2S have already been discussed earlier. However, randomized controlled trials (RCTs) in UC patients that evaluated the efficacy of prebiotic supplementation alone demonstrated limited weak effect[39] which indicates that a multi-prong approach, not just prebiotic supplementation, is required to achieve clinical effects.

Secondly, there is some evidence from epidemiological studies that provide clues for the influence of dietary sulfur-containing protein, sulfates and sulfites on the clinical course of UC. One study reported a correlation between a high protein intake and increased risk of developing UC[40] whilst only one study has shown an association between a high intake of sulfur amino acids and sulfate with a three-fold greater risk of disease relapse[41]. Subsequently, the potential clinical efficacy of a sulfur-restricted diet was first described from a small open-label study in eight UC patients. The low sulfur diet combined with stable salazopyrin therapy was associated with histological and clinical improvement[9]. Changes in colonic H_2S production was unfortunately, not measured as a mechanism for efficacy but the promise of this dietary approach warrant further investigation in a controlled trial.

Furthermore, there has been growing interest in the role of carrageenan, a sulphated polysaccharide food additive that escapes digestion in the small intestine almost intact and is fermented to release sulphates[42], which is then metabolised to produce H_2S. In 2017, a RCT by Bhattacharyya *et al*[43] assessed the role of carrageenan, a sulphated polysaccharide food additive, in maintaining relapse in 14

UC patients in remission. Following a year of no-carrageenan diet, relapse rates appeared to be higher in the five patients receiving 200 mg carrageenan, a dose slightly below average intakes of carrageenan in the US diet, than those who received placebo capsules. Unfortunately, significant recruitment issues impacted on the sample size of the study, making it difficult to ascertain whether this was a real clinical effect.

Finally, the major sources of nitrites in the diet are food preservatives in cured and processed meat, while the major source of dietary nitrates is vegetables[44]. Thus far, the effect of different sources of nitrite and nitrate in the diet on microbial NO production are unknown and should be further investigated in clinical studies.

TRANSLATING PROPOSED DIETARY STRATEGY INTO CLINICAL APPLICATION

Several dietary strategies can therefore be implied for future clinical application from studies thus far. First, a multi-prong intervention targeting dietary substrates with H_2S-modulating abilities, expanding on Roediger[9]'s earlier work, is warranted in active UC patients. This approach should consider reducing intake of sulfur-containing protein such as methionine, cysteine and taurine, and added sources of inorganic sulfur to reduce excessive/chronic H_2S exposure to the colonic epithelium. Major sources of these foods are listed in Table 1. Inorganic sulfur exist as food additives in several forms, sulfur dioxide (E220), sulfites (E221-E227) and as a sulphated polysaccharide, carrageenan (E407). In Australia and Europe, food labelling requirements mandate only the labelling of added sulphites (in amounts > 10 mg/kg) in food product without specifying the amount used. Inorganic sulfur intake by foods and beverages has been showed to be six-fold higher in the western diet in comparison to a typical African rural diet[45,46]. However, food composition tables on sulfur-containing protein, inorganic sulfur and carrageenan are far from complete to adequately assess habitual intake of UC patients, to ensure successful design of the dietary therapy. More importantly, there are grounds that increasing a combination of fibre, rather than restricting total fibre, maybe an efficacious strategy for H_2S suppression whilst improving nutrient delivery to the colonic epithelium in UC. Resistant starch and fructo-oligosaccharides, whilst efficacious, are fermented in the proximal colon[10]. Hence, a strategy that will carry the fermentation of these fibres across the entire colon by combining with a minimally fermentable fibre is required.

Indeed, tolerability and the potential clinical effects of a dietary approach incorporating strategies discussed above have already been evaluated. In an open-label dietary advice study, Day *et al*[47] reported excellent tolerability of dietary strategy called the 4-strategies to a sulphide-reducing (4-SURE) diet by patients with mild to moderately active UC. This was despite the 38% increase in dietary fibre and the four-fold increase in resistant starch intake by these patients. Food-related quality of life also increased markedly. Whilst it was impractical to assess colonic H_2S in this study, markers of protein fermentation, namely faecal branched chain fatty acids were used as a surrogate. The significant reduction in faecal branched to short-chain fatty acid ratio following the 4-SURE study indicated that protein fermentation being the major pathway for luminal H_2S production was reduced. Whilst the study did not intend to primarily assess clinical end-points due to the uncontrolled study design, there were indicators for the diet to positively affect clinical outcomes and mucosal healing. Data supported by a significant reduction in faecal calprotectin. A second dietary approach, called the Ulcerative Colitis Exclusion Diet (UCED), also incorporated a similar exclusion of decreasing intake of total protein, sulphur-containing amino acids, food additives along with additional restrictions of animal and saturated fat, haeme, whilst increasing intake of tryptophan, pectin and resistant starch[48]. In a RCT comparing a combination of UCED with faecal transplant, faecal transplant or diet alone, Sarbagili Shabat *et al*[48] observed that clinical response and endoscopic remission were the greatest for the UCED diet. Furthermore, the promising outcomes of the UCED was supported by an earlier open-label study in paediatric patients with mild to moderate active UC on stable maintenance therapy, where the diet treatment showed that patients had a significant decrease in sulfur-containing amino acids consumption as well as a significant increase in total fiber consumption[49].

Whilst these proposed dietary approaches are not quite ready for clinical application until RCTs have been performed (currently underway) to replicate the promising findings, it does suggest that patients with active inflammation do tolerate a certain increase in high fibre foods and builds on the suggestion to minimise intake of processed foods. Moreover, the limitations of the available reported clinical trials targeting reduction of H_2S production as a treatment strategy for UC (Table 2) suggest the need for larger, high quality dietary studies incorporating gut microbiome composition and function assessment including changes in microbial H_2S metabolism.

BIOMARKERS FOR ASSESSING RESPONSE OF DIETARY THERAPY

It is key that a biomarker for assessing diet response is incorporated early on after dietary therapy is administered as a way of assessing whether the diet is achieving its intended mechanistic effect. An

Table 1 Content of sulfur, nitrate and nitrite in selected foods

Food category	Specific food	Sulfur amino acids (cysteine + methionine)[1], mg/100 g	Sulfates[2], mg/100 g/mL	Nitrate[3], mg/kg	Nitrite[3], mg/kg
High sulfur amino acids foods	Beef	239	-	-	-
	Chicken	291	-	-	-
	Turkey	269	-	-	-
	Tuna	268	-	-	-
	Prawns	189	-	-	-
	Eggs	162	-	-	-
	Cheese, hard	174	-	-	-
High sulfites foods	Dried apricots	-	300	-	-
	Dried apples	-	490	-	-
	Commercial bread	-	80-150	-	-
	Wine	-	38	-	-
High sulfates foods	Cabbage	-	84	-	-
	Broccoli	-	90	-	-
	Cauliflower	-	50	-	-
	Brussels sprouts	-	93	-	-
High nitrates foods	Lettuce	-	-	2351	-
	Celery	-	-	2110	-
	Spinach	-	-	1509	-
	Leek	-	-	841	-
High nitrites foods	Sausages, boiled	-	-	-	40
	Poultry meat	-	-	-	32
	Beef	-	-	-	59
	Bacon	-	-	-	86

[1]From reference Magee *et al*[46], 2004.
[2]From reference Florin *et al*[45], 1993.
[3]From reference Temme *et al*[44], 2011.

example of this is the reduction in breath hydrogen production after introduction of a diet low in fermentable carbohydrates as a biomarker of intervention success[50]. However, in the case of dietary approaches targeting colonic H_2S and NO, there are difficulties with accessing reliable measurement techniques for these volatile gases, particularly with *ex vivo* measurements often requiring freshly passed faecal samples[17,33], which introduces practical issues for trial patients. Currently, measurements for H_2S mainly involve faecal sulphide, urinary sulphate or breath H_2S[51]. Sensitivity of these measurements are impacted by its adsorption or susceptibility to oxidation, yielding low concentrations[51]. In contrast, the only reported assessment of luminal NO has been using direct sampling (*via* a tonometric balloon) and measurement *via* a rapid-response chemiluminescence technique[21]. While the method has good sensitivity, it is unknown whether this biomarker is directly responsive to alterations in dietary nitrate and nitrite intake. Finally, there is potential for direct intestinal gas sampling, such as the gas-sensing capsule[52], but these do not yet measure H_2S or NO. In the absence of reliable direct measurements, indirect assessments could target markers of protein fermentation for H_2S, quantification of sulphate- or sulphite-reducing bacteria which are dependent on availability of proteinaceous substrates for growth[53], and have capacities for denitrification and sulphate-reduction [54]. Hence, an effective biomarker for monitoring the success of sulphide- and NO-reducing dietary approaches remains elusive and is very much needed to support the development of the proposed dietary therapies. Therefore, as in most studies, assessment of dietary response is primarily assessed by different questionnaires such as dietary intake questionnaires, food-related quality of life or health-related quality of life questionnaire. Combined biomarker measurements with assessment by question-

Table 2 Summary of studies clinical outcomes by dietary interventions for ulcerative colitis as a possible strategy to modify hydrogen sulfide production

Ref.	Dietary intervention	Study design	Main outcomes	Limitations
Roediger[9], 1998	Low sulfur diet	Open-label, prospective pilot study. Patients were instructed to follow low sulfur diet + stable dose of salazopyrin for 12 mo (n = 4 adults)	All patients showed clinical and histological improvement and no relapse attacks were observed	Very small sample size
Bhattacharyya *et al*[43], 2017	No-carrageenan diet	Double-blind RCT: Carrageenan capsules versus placebo. Patients with remission were followed up until relapse or of 12 mo (n = 12 adults)	The carrageenan group demonstrated significant higher relapse rate and an increase in FC and IL-6 values from study onset	Small sample size in each group. The effects on the microbiome were not addressed and precise measurements of compliance with the diet were not performed
Chiba *et al*[58], 2019	Lacto-ovo-semivegetarian diet-PBD	Prospective single arm study. Patients were followed after induction therapy incorporating PBD (n = 92 children and adults)	The cumulative relapse rates at 1 and 5 yr were 14% and 27% respectively, which is indicated by the authors to be lower than those previously reported	Small sample size without control group. The mechanistic effect of the diet was not addressed
Sarbagili Shabat *et al*[48], 2022	UCED	Single-blind RCT in adults with active refractory UC: Group1: FT alone; group2: FT with UCED; group3: UCED alone. The primary endpoint was week 8 clinical remission (n = 51)	UCED alone demonstrated the greatest clinical and endoscopic remission rates compared to single donor FT with or without diet	Small sample size in each group. Eligibility criteria include patients with severe UC, of whom none obtain remission. The effects on the microbiome were not addressed
Sarbagili-Shabat *et al*[49], 2021	UCED	Open-label, prospective pilot study in children with active UC. The primary endpoint was week 6 clinical remission (n = 24)	UCED lead to 38% clinical remission and FC improvement	Small sample size without control group. The effects on the microbiome were not addressed
Day *et al*[47], 2022	4-SURE	Open-label, prospective pilot study in adults with active UC. The primary endpoint was week 8 tolerability (n = 28)	The 4-SURE diet was well tolerated and lead to 46% clinical response and 36% endoscopic improvement. Fecal excretion of SCFAs increased while BCFAs decreased	Changes in colonic H_2S not able to be measured. Lack of control and inadequate power for interpretation of secondary clinical end-points

RCT: Randomized controlled trial; FC: Fecal calprotectin; IL-6: Interleukin-6; PBD: Plant-based diet; UCED: Ulcerative colitis exclusion diet; FT: Faecal transplantation; UC: Ulcerative colitis; 4-SURE: 4 Strategies to SUlfide-Reduction; SCFA: Short chain fatty acid; BCFA: Branched chain fatty acid; H_2S: Hydrogen sulfide.

naires can be the ideal tool for estimating the effect of specific dietary exposure.

CONCLUSION

Microbial H_2S and NO metabolites have causative roles in the pathogenesis of UC *via* their damaging effects on the colonic epithelium. Modulation of their production within the colonic lumen in order to reduce colonic epithelial exposure to these luminal stressors presents an attractive therapeutic target that has yet to be adequately explored. The current evidence suggests that dietary manipulation is likely to be an effective strategy to modify colonic H_2S production whereas little is known regarding dietary modulation of NO. It is also clear that sulfur-containing amino acids are major substrates that promote H_2S production over inorganic sulphur but data has emerged suggesting that increasing fermentable fibre is highly efficacious in reducing H_2S production. These findings have been utilised to inform the design of multi-prong dietary approaches which have yielded promising therapeutic efficacy in mild to moderate active UC. However, key to advancing the success of this research is the urgent need for better technology to accurately assess luminal concentrations of these volatile gases. Finally, before implementation into dietary practice can be pursued, further investigations into their efficacy on altering disease activity using robust dietary trial designs (which are currently underway), expansion of food composition data and mechanisms of H_2S reduction are highly warranted.

FOOTNOTES

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She also works in a department that financially benefits from the sales of a digital application and booklets on the low fermentable oligosaccharides, disaccharides, monosaccharides and polyols diet. Funds raised contribute to research of the Department of Gastroenterology and to the University. She does not receive personal remuneration. Sarbagili-Shabat C has no conflicts of interest to report.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Disease trends after *Helicobacter pylori* eradication based on Japanese nationwide claims and the health check-up database

Kazuhiro Mizukami, Kentaro Sugano, Tomomi Takeshima, Kazunari Murakami

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Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) infection is a risk factor for many diseases, including peptic ulcer disease and gastric cancer. While *H. pylori* eradication therapy can prevent these diseases, potentially unfavorable effects of eradication therapy have also been reported in some diseases, such as gastroesophageal reflux disease (GERD), Barrett's esophagus (BE), inflammatory bowel disease (IBD), allergic diseases, and metabolic diseases. Consequently, both positive and negative impacts should be considered when assessing the effects of *H. pylori* eradication therapy.

AIM

To compare the incidence of these diseases before and after *H. pylori* eradication and to comprehensively assess its effects.

METHODS

This retrospective cohort study used a Japanese nationwide health claims database (April 2009-March 2020), developed by the Japanese Ministry of Health, Labour and Welfare. The database contained almost all health insurance claims data issued in Japan, and specific health check-up data for individuals who took the check-ups. Descriptive statistics were used for the analyses. Patients who received primary eradication therapy were defined as those prescribed medication for *H. pylori* eradication. New diagnoses, defined as incidence of upper gastrointestinal diseases and IBD, and prevalence of allergic diseases were compared before and after eradication. The incidence and prevalence of each disease were also compared between the 3-year period before eradication (from the 4th to the 2nd year prior to the year of eradication) and the 3-year period after

eradication (from the 1st to the 3rd year after the year of eradication) based on the age category and calendar year and month. Changes in body mass index and proportion of patients with metabolic syndrome (MS) were examined before and after eradication.

RESULTS

We identified 5219731 patients who received primary eradication therapy. The 65-69 years age group had the greatest number of patients in both sexes. There was no significant increase in the incidence of GERD after eradication when considering the effects of aging and reporting period. However, the incidence of BE was higher in the 3-year period after eradication than in the 3-year period before eradication for all age categories (0.02%-0.10% *vs* < 0.01%-0.05%). The incidence of IBD and prevalence of allergic disease were also higher after eradication. In contrast, the incidence of gastric and duodenal ulcers and gastritis was reduced after eradication. In patients with at least one entry of health check-up data (1701111 patients), the percentage of patients with MS showed a slight increase following eradication (11.0% in the year of eradication and 12.2% after 5 years).

CONCLUSION

The results suggest that *H. pylori* eradication therapy reduces peptic ulcers and gastritis; however, it is associated with increased incidence of several other chronic diseases.

Key Words: Administrative claims; Healthcare; Allergy; Eradication therapy; Gastroesophageal reflux disease; *Helicobacter pylori*; Inflammatory bowel disease

Core Tip: While *Helicobacter pylori* (*H. pylori*) eradication can prevent certain diseases including peptic ulcer diseases and gastric cancer, unfavorable effects of eradication therapy have also been reported. We analyzed a Japanese nationwide health claims database containing almost all health insurance claims data to compare the incidence and prevalence of specific diseases before and after *H. pylori* eradication to comprehensively assess its effects. We identified 5219731 patients who received primary eradication therapy. *H. pylori* eradication drastically reduced peptic ulcers and gastritis but was associated with an increase in Barrett's esophagus, inflammatory bowel disease, allergic disease, and metabolic syndrome.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is a risk factor for many diseases, including peptic ulcers[1], gastric cancer[2], gastric mucosa-associated lymphoid tissue lymphoma[3], *H. pylori*-associated gastritis[4], idiopathic thrombocytopenic purpura[5], and iron deficiency anemia[6]. *H. pylori* eradication therapy can effectively prevent these diseases. However, unfavorable effects of eradication therapy have also been reported. For instance, the relationship between *H. pylori* infection and gastroesophageal reflux disease (GERD) is controversial. Some studies have suggested a lower incidence of GERD and its complications, such as Barrett's esophagus (BE), in patients with *H. pylori* infection[7-10]. A study reported that the prevalence of GERD increased in patients after successful *H. pylori* eradication and was comparable to that in patients without *H. pylori*[11]. However, some studies found no association between *H. pylori* eradication and GERD[12], while others reported that eradication is not associated with GERD development in dyspeptic patients but might pose a higher risk in patients with peptic ulcers[13]. An inverse association of *H. pylori* infection with some immune system diseases, including allergies[14-18] and inflammatory bowel disease (IBD)[18-22], has also been reported. Moreover, relationships between *H. pylori* infection and metabolic diseases and associated conditions such as obesity have also been described. *H. pylori* infection may increase the risk of dyslipidemia[23,24] and metabolic syndrome (MS)[25]. While favorable effects of *H. pylori* eradication on triglyceride and high-density lipoprotein cholesterol (HDL-C) levels[26] or dyslipidemia[27] have been reported, it has also been found that eradication therapy may increase the incidence of hyperlipidemia and obesity[28,29]. Therefore, both positive and negative impacts should be considered when assessing the effects of *H.*

pylori eradication therapy. A comprehensive evaluation of these effects in Japan, where large-scale eradication therapy was commenced earlier than it was in other countries due to its coverage by universal healthcare, might provide useful information for clinicians worldwide.

Herein, we performed a comprehensive analysis of the effects of *H. pylori* eradication therapy on various diseases. This study used a Japanese nationwide health claims database, the National Database of Health Insurance Claims and Specific Health Checkups of Japan (NDB), developed by the Japanese Ministry of Health, Labour and Welfare, containing almost all (≥ 95%) health insurance claims data issued in Japan[30]. In this database, health check-up data are also included for individuals who underwent specific health check-ups. We compared the development of diseases (defined as the first diagnosis) known to be related to *H. pylori* infection and eradication before and after eradication in patients who underwent primary *H. pylori* eradication therapy. We also assessed changes in body mass index (BMI) and MS development before and after eradication.

MATERIALS AND METHODS

Study design and data source

This claims-based study used NDB data[30] (April 2009-March 2020). The dataset contained medical claims data, diagnosis procedure combination (DPC) claims data (claims related to bundled payments during DPC hospitalization), and pharmacy claims data from patients who received primary *H. pylori* eradication therapy or those who had at least one diagnosis of GERD. Specific health check-up data available for patients who underwent check-ups included anthropometric data, laboratory values, and answers to questionnaires. The observation period was defined as the entire period of data availability for each patient.

Patients

Patients with a prescription for primary *H. pylori* eradication therapy covered by national health insurance were identified and designated in our study as those who received primary *H. pylori* eradication therapy. The three medications approved for this therapy are amoxicillin and clarithromycin with either a proton pump inhibitor (PPI) or potassium-competitive acid blocker (P-CAB); these can be prescribed as a combination of single drugs in the same month or as a combination pack (Supplementary Table 1).

Outcomes and analysis

We compared the incidences of GERD, BE, other upper gastrointestinal diseases [including some diseases of the digestive system other than those categorized as International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10) code K92], and IBD, and the prevalence of allergic diseases before and after primary *H. pylori* eradication therapy (see Supplementary Table 2 for definition of the diseases). Furthermore, IBD was classified into Crohn's disease (CD) and ulcerative colitis (UC) according to the ICD-10 classification. Other upper gastrointestinal diseases were classified into 10 types [esophagitis, other diseases of the esophagus, gastric ulcer (GU), duodenal ulcer (DU), peptic ulcer, site unspecified, anastomotic ulcer, gastritis and duodenitis, functional dyspepsia (FD), other diseases of the stomach and duodenum, and other diseases of the digestive system] according to the ICD-10 classification, and allergic diseases were classified into five types (allergy, pollinosis, asthma, hypersensitivity, and atopy) by disease name (Supplementary Table 2). It should be noted that since allergic diseases were initially defined according to the ICD-10 classification and atopic asthma was classified into the asthma group, the atopy group included only atopic cough. The month of primary eradication therapy was defined as the earliest month in which primary eradication medications were prescribed for each patient. The term 'year of eradication' refers to the year corresponding to this month. The incidence of a disease was calculated on the basis of the earliest first diagnosis of each disease, which was identified using the record of the first diagnosis date by the medical institution on medical claims or DPC claims in the database.

The incidence and prevalence of each disease was calculated for each elapsed year from the eradication, which was calculated as the integer part of the difference of the time and the time of the eradication. If the difference was negative, the elapsed year was the integer part minus one, as in SAS (version 9.4, SAS Institute, Cary, NC), the integers of the negative numbers were rounded up.

We also compared the incidence and prevalence of each disease between the 3-year period before eradication (from the 4th to 2nd year prior to the year of eradication) and the 3-year period after eradication (from the 1st to 3rd year after the year of eradication) based on the age category and calendar year and month, represented by calendar month and year from July 2014 to June 2018.

To assess GERD status in patients with and without *H. pylori* eradication, we compared treatments in patients who received eradication therapy before GERD development with those who did not undergo eradication therapy during the observation period. Patients with available health check-up data for the year of the first GERD diagnosis were included in this analysis (eradication group), and the data were used to develop a propensity score (PS). Patients in the non-eradication group were identified from the

patients with GERD, those who did not undergo eradication therapy, and those who had their health check-up data. The patients identified in the non-eradication group were those whose sex, age at the first GERD diagnosis, and the month of the first GERD diagnosis matched with those of anyone in the eradication group. After adjusting for confounding factors using the PS, the types of drugs prescribed for GERD were compared by year from first GERD diagnosis and by calendar year between the eradication and non-eradication groups. The PS was estimated using a logistic regression model with eradication (dummy variable) as the explained variable, and age, sex, and specific health check-up data (BMI, abdominal circumference, systolic blood pressure, diastolic blood pressure, triglyceride, HDL-C, low-density lipoprotein cholesterol, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, fasting blood glucose, and hemoglobin A1c) as explanatory variables. The PS was divided into four classes, and patients within each group were assigned the same weight to make the total weight comparable between groups in each PS class. The following drug classes for GERD treatment were analyzed: Histamine H_2 receptor antagonist, P-CAB, PPI, and others (Supplementary Table 3). In patients who received GERD treatment in the month of first diagnosis, the period of continuous GERD treatment was compared between patient groups after adjustment using the Kaplan-Meier method with the log-rank test (in this test, $P < 0.05$ was considered statistically significant). Treatment was defined as any treatment with no gap exceeding 3 mo.

Changes in BMI and proportion of patients with MS were examined before and after eradication in patients for whom these data of specific health check-up were available at least once or yearly for the 3 years before and after the year of eradication. If multiple values were recorded for a patient in the same year, the average value was used. Patients with MS were defined as those with an abdominal circumference of ≥ 85 cm in men or ≥ 90 cm in women, and who met at least two of the following criteria: (1) Triglyceride ≥ 150 mg/dL or HDL-C < 40 mg/dL; (2) Systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg; and (3) Fasting plasma glucose ≥ 110 mg/dL[31]. We used the statistical software packages SAS for the analyses. In the analyses using the specific health check-up data, missing values were imputed based on all other factors using the MI statement in PROC MI of the SAS software.

Ethical approval

This study was approved by the Ethics Committee of Oita University, Faculty of Medicine (No. 1692). All procedures followed were in accordance with the World Medical Association's Declaration of Helsinki (1964, and its later amendments). Informed consent was not obtained because this study used anonymized claims data.

RESULTS

Overview of disease developments

The dataset included 5219731 patients who received primary *H. pylori* eradication therapy. Maximum number of patients were in the 65-69 years age bracket at the time of eradication. There were more men aged < 65 years, but more women aged ≥ 65 years (Figure 1).

The incidences of upper gastrointestinal diseases and IBD were highest in the year of eradication and the preceding year, respectively (Table 1). After eradication, the incidences of GERD and IBD decreased yearly, reaching similar levels to those before eradication. The incidences of other upper gastrointestinal diseases markedly decreased after eradication. In contrast, the incidence of BE increased after eradication. The prevalence of allergic diseases increased in the years before eradication and continued to increase after eradication; the prevalence at 5 years after eradication was almost double the prevalence approximately 10 years before eradication (Table 1).

GERD

The incidence of GERD in the 3-year period before eradication was similar to or slightly higher than that in the period after eradication for all age groups, and it tended to increase with age regardless of eradication status (Figure 2A). When data were analyzed according to the calendar year and month, the incidence was higher after eradication, although this difference disappeared in later years (Figure 2B).

When comparing GERD treatment status between the eradication group (76367 patients) and non-eradication group (1008539 patients), there was no remarkable difference in the trend of GERD medication by year from first GERD diagnosis or calendar year, although the percentage of patients receiving each type of GERD drug was slightly higher in the eradication group (Supplementary Figure 1). In patients who received GERD medication in the month of the first GERD diagnosis (72170 and 920794 patients in the eradication and non-eradication groups, respectively), the continuous treatment period was significantly longer in the eradication group ($P < 0.01$) (Supplementary Figure 2).

BE

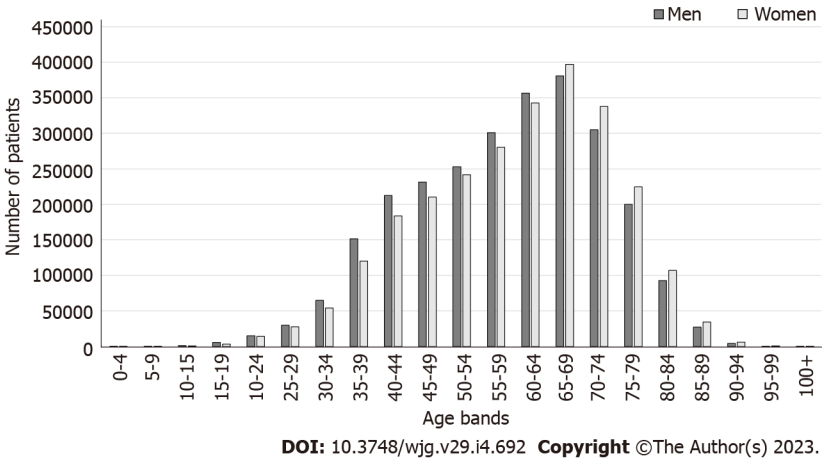
The incidence of BE was higher in the 3-year period after eradication than in the period before eradication for all age categories (Figure 3A), and throughout the observation period (Figure 3B). The

Table 1 Incidence or prevalence of each disease by year from primary *Helicobacter pylori* eradication therapy

Year	Denominator (patient-year)	Average age	Upper gastrointestinal diseases			IBD	Allergy
			GERD	Barrett's esophagus	Other upper gastrointestinal diseases		
-10	1146497	48.9	2.31% (2.28%, 2.33%)	0.004% (0.002%, 0.005%)	11.45% (11.39%, 11.51%)	0.029% (0.026%, 0.032%)	17.5% (17.4%, 17.5%)
-9	1943505	50.1	2.55% (2.52%, 2.57%)	0.006% (0.005%, 0.008%)	11.10% (11.05%, 11.14%)	0.028% (0.025%, 0.030%)	20.0% (19.9%, 20.0%)
-8	2761765	51.3	2.75% (2.73%, 2.77%)	0.007% (0.006%, 0.008%)	10.57% (10.54%, 10.61%)	0.026% (0.024%, 0.028%)	21.3% (21.2%, 21.3%)
-7	3364755	52.5	3.02% (3.00%, 3.04%)	0.010% (0.009%, 0.011%)	9.89% (9.86%, 9.92%)	0.027% (0.025%, 0.029%)	23.1% (23.1%, 23.2%)
-6	4001760	53.7	3.18% (3.16%, 3.19%)	0.011% (0.010%, 0.012%)	9.27% (9.24%, 9.30%)	0.027% (0.025%, 0.028%)	24.4% (24.3%, 24.4%)
-5	4520587	54.9	3.38% (3.37%, 3.40%)	0.015% (0.014%, 0.016%)	8.67% (8.64%, 8.70%)	0.028% (0.026%, 0.029%)	25.7% (25.7%, 25.7%)
-4	4746859	55.9	3.48% (3.47%, 3.50%)	0.018% (0.017%, 0.019%)	7.54% (7.51%, 7.56%)	0.027% (0.025%, 0.028%)	27.4% (27.3%, 27.4%)
-3	4880888	56.9	3.58% (3.56%, 3.59%)	0.023% (0.022%, 0.024%)	6.50% (6.48%, 6.53%)	0.027% (0.026%, 0.028%)	28.8% (28.7%, 28.8%)
-2	5025747	57.9	3.80% (3.78%, 3.81%)	0.030% (0.028%, 0.031%)	6.04% (6.02%, 6.06%)	0.029% (0.027%, 0.030%)	30.3% (30.3%, 30.4%)
-1	4732998	58.8	11.01% (10.99%, 11.04%)	0.164% (0.161%, 0.168%)	19.31% (19.27%, 19.35%)	0.046% (0.044%, 0.048%)	33.7% (30.3%, 30.4%)
0	4892585	59.8	11.33% (11.30%, 11.36%)	0.223% (0.219%, 0.227%)	16.48% (16.45%, 16.51%)	0.053% (0.051%, 0.055%)	34.7% (34.7%, 34.8%)
1	4137930	61.0	3.84% (3.82%, 3.86%)	0.083% (0.080%, 0.086%)	0.15% (0.15%, 0.16%)	0.034% (0.032%, 0.036%)	35.0% (34.9%, 35.0%)
2	3344893	62.2	3.30% (3.28%, 3.32%)	0.080% (0.077%, 0.083%)	0.12% (0.12%, 0.13%)	0.030% (0.028%, 0.032%)	35.7% (35.7%, 35.8%)
3	2520763	63.4	2.98% (2.96%, 3.00%)	0.076% (0.072%, 0.079%)	0.10% (0.09%, 0.10%)	0.030% (0.028%, 0.032%)	36.7% (36.7%, 36.8%)
4	1900509	64.5	2.76% (2.74%, 2.78%)	0.076% (0.072%, 0.080%)	0.09% (0.08%, 0.09%)	0.028% (0.026%, 0.031%)	36.5% (36.4%, 36.6%)
5	1275881	65.4	2.51% (2.49%, 2.54%)	0.073% (0.069%, 0.078%)	0.07% (0.07%, 0.07%)	0.028% (0.025%, 0.031%)	38.6% (38.5%, 38.7%)

The prevalence of allergic diseases and incidence of the other diseases are shown with 95% confidence intervals (lower limit, upper limit). Year 0 is the year of primary eradication, and negative values represent years before primary eradication. The elapsed year from the eradication was calculated as the integer part of the difference in the year and the time of the eradication. If the difference was negative, the elapsed year was the integer part minus one, as in SAS, the integers of the negative numbers were rounded up. GERD: Gastroesophageal reflux disease; IBD: Inflammatory bowel disease.

incidence of BE increased with age regardless of *H. pylori* eradication status in patients < 70 years of age and decreased thereafter beyond the age of 70 years (Figure 3A).



DOI: 10.3748/wjg.v29.i4.692

Figure 1 Number of patients who received primary *Helicobacter pylori* eradication therapy, by sex and age group at the time of eradication.

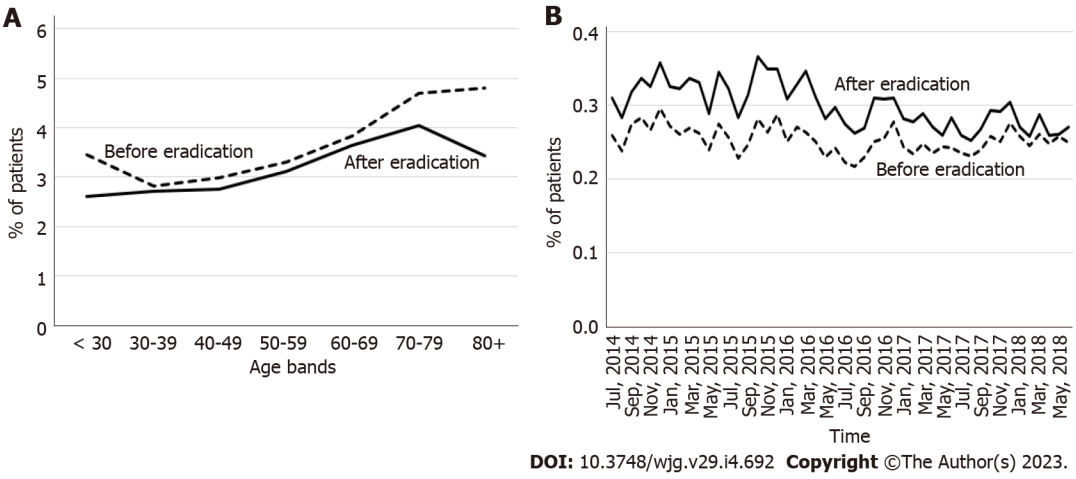


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Figure 2 Incidence of gastroesophageal reflux disease in the 3-year period before the year of eradication and after the year of eradication. A: By age categories; B: By calendar year and month.

Other upper gastrointestinal diseases

The incidence of other upper gastrointestinal diseases was lower in the 3-year period after eradication than in the period before eradication for all age categories (Supplementary Figure 3A), and throughout the study period (Supplementary Figure 3B).

Gastritis and duodenitis showed the highest incidences before eradication, and their incidences decreased after eradication (Supplementary Figure 4). The incidences of GU and DU were also higher before eradication than after eradication (Supplementary Figure 4). The incidences of other diseases, including FD, esophagitis, and other diseases of the esophagus, increased with time and were higher after eradication than before (Supplementary Figure 4). Of note, the overall incidence of these diseases was lower than that of the other upper gastrointestinal diseases.

IBD

The incidence of CD was higher in the 3-year period after eradication than in the period before eradication for younger age categories. This difference decreased with increasing age, particularly for patients aged ≥ 60 years (Figure 4A). The incidence of UC was higher in the 3-year period after eradication than in the period before eradication for patients aged ≥ 30 years (Figure 4B). For both diseases, the incidence tended to decrease with increasing age up to 50-59 years; the incidence of CD was very low for this and older age groups (Figures 4A and B).

Allergic diseases

The prevalence of allergic diseases was higher in the 3-year period after eradication than in the period before eradication for all age categories (Figure 5A), and throughout the entire study period (Figure 5B).

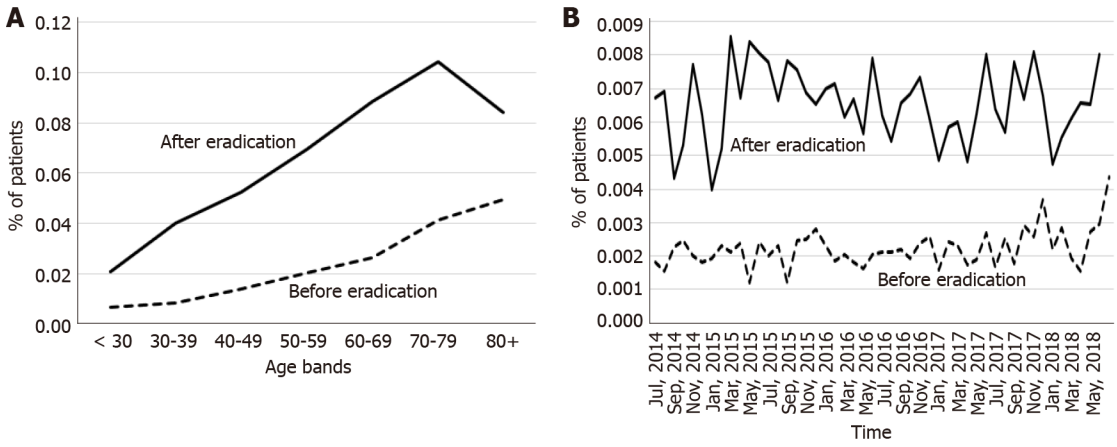

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Figure 3 Incidence of Barrett's esophagus in the 3-year period before the year of eradication and after the year of eradication. A: By age category; B: By calendar year and month.

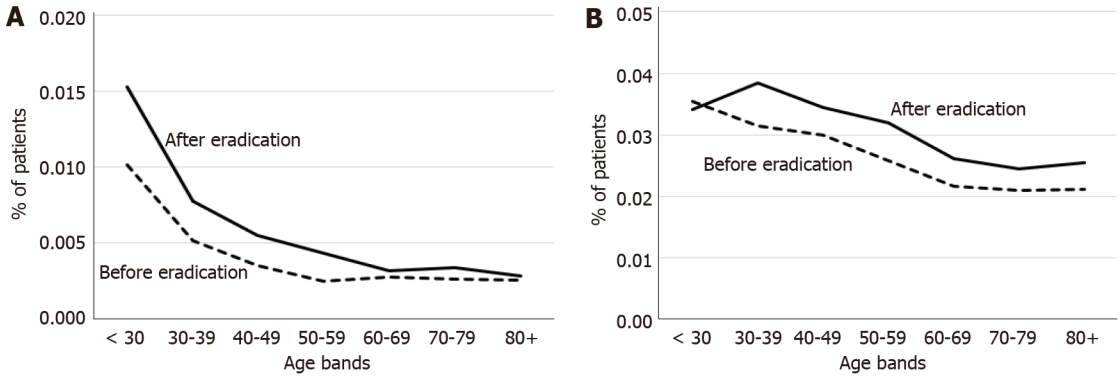

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Figure 4 Incidence of inflammatory bowel disease in the 3-year period before the year of eradication and after the year of eradication by age category. A: Crohn's disease; B: Ulcerative colitis.

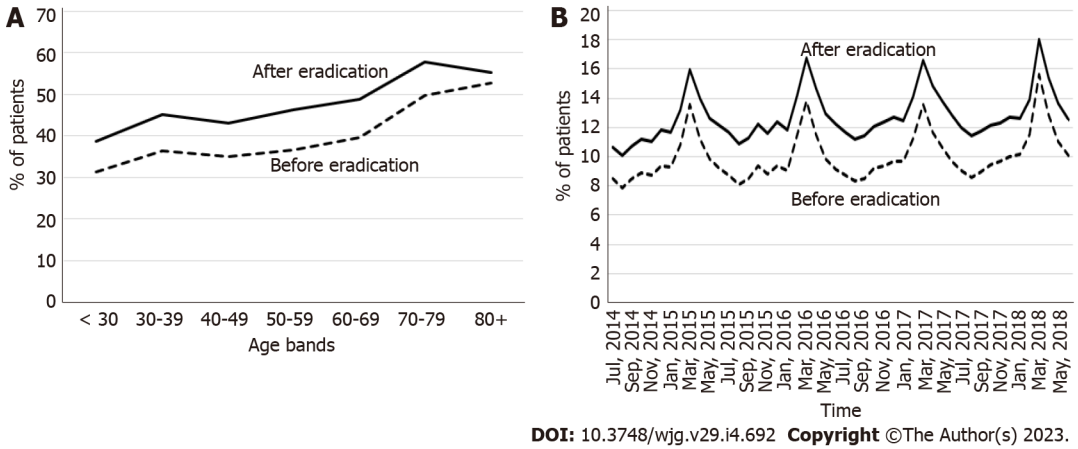

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Figure 5 Prevalence of allergic diseases in the 3-year period before the year of eradication and after the year of eradication. A: By age category; B: By calendar year and month.

The prevalence of allergic diseases tended to be higher in older patients (Figure 5A); moreover, periodic seasonal changes were observed, with a higher incidence in early spring (Figure 5B), both before and after eradication. The prevalence of all types of allergies increased after eradication (Supplementary Figure 5).

Changes in BMI and MS

At least one set of data regarding BMI was available for 1701111 patients during the observation period, and 100954 patients had BMI data available for every year of the 3 years before and after the year of eradication. Among patients with at least one data point, the average BMI slightly decreased toward the year of eradication, following which it slightly increased (Figure 6A). This increase was even more remarkable among those with complete BMI data in the 3 years before and after eradication.

In all patients with health check-up data available at least once (1701111 patients), the percentage of patients with MS slightly increased following eradication (Figure 6B). An even larger increase after eradication was observed in patients with all data from 3 years before and after eradication.

DISCUSSION

This study comprehensively analyzed the effects of *H. pylori* eradication, including both favorable and unfavorable aspects, in Japan. The database used in this study is a nationwide claims database comprising data from almost the entire Japanese population, including more than 5 million people who received primary *H. pylori* eradication therapy. While favorable effects on GU, DU, and gastritis were confirmed, the treatment could result in the development of several concerning diseases. In particular, the possibility of an increase in allergic diseases is a new finding that has not been reported in the past.

When the effect of aging and reporting period was considered, there was no significant increase in the incidence of GERD after eradication. Previous studies reported conflicting results regarding the relationship between *H. pylori* infection and GERD development. Among Japanese *H. pylori*-positive patients, the odds ratio (OR) for GERD development is reported to be 0.35-0.50[10,32,33]. The lower rates of GERD development in patients with *H. pylori* is reportedly due to the suppression of gastric acid production caused by *H. pylori* infection. However, a systematic review based on randomized controlled trials (RCTs) of patients who underwent *H. pylori* eradication indicated no significant increase in GERD occurrence at 6, 12, and 24 mo following eradication[12]. No significant effects of *H. pylori* eradication on GERD development were suggested by another systematic review analyzing RCTs[34]. In addition, another RCT showed that *H. pylori* eradication did not affect the recurrence rate of GERD after eradication[35]. In contrast, an increased risk following eradication therapy was suggested by a meta-analysis of cohort studies and RCTs[36]. These contradictory results might be due to differences in baseline diseases and study design. In fact, another systematic review suggested that eradication is not associated with GERD development in dyspeptic patients, whereas an association in patients with peptic ulcers is suggested by results of cohort studies but not of RCTs[13]. Collectively, the findings of previous reports together with our results suggest the lack of an association between *H. pylori* eradication and GERD development. Moreover, the type of GERD medication did not differ between patients with and without eradication. This suggests that a change in GERD treatment including a switch to more powerful P-CAB or an increase in dosage of PPI is not required following eradication.

After eradication, a higher incidence of BE was observed in all age groups and over time. A previous case-control study reported a lower risk of BE in patients with *H. pylori* infection, with an overall OR of 0.55 [95% confidence interval (CI): 0.35-0.84] after adjusting for age and white race, as well as an OR of 0.28 (0.15-0.50) in those with corpus atrophy or antisecretory drug use[37]. The diagnostic criteria of BE is different in Japan and Western countries, so direct comparisons are difficult[38]. However, a possible explanation for the higher incidence of BE may have to do with the palisade vessel at the distal esophagus, a landmark of the gastroesophageal junction used in Japan, being more clearly identifiable by endoscopy after eradication, as *H. pylori* may colonize the esophageal mucosa, causing inflammation [39,40]. After eradication of *H. pylori*, small areas of columnar metaplasia may become visible due to resolution of mucosal inflammation. Utilization of advanced image-enhanced endoscopy, such as linked color imaging may also contribute to a higher diagnostic performance for BE[41]. Notably, BE lesions in Japanese patients were very short (< 1 cm) in most cases, and patients with ≥ 3-cm lesions were rare (approximately ≤ 1%)[42]. A previous Japanese case report described a case of esophageal adenocarcinoma after *H. pylori* eradication[43]. However, a Swedish cohort study indicated no evidence of an increased risk of BE or esophageal adenocarcinoma after *H. pylori* eradication[44]. Further research, including an evaluation of the characteristics of BE and its association with cancer, is warranted to evaluate the true effects of eradication on BE in Japanese patients.

Both age-dependent and monthly analyses suggest lower incidences of other upper gastrointestinal diseases following eradication therapy. Among these diseases, GU, DU, and gastritis and duodenitis (analyzed together) were the most common before eradication. The effect of *H. pylori* eradication on the risk of GU, DU, and gastritis has been previously established[1,4]; therefore, the reduced incidence of these diseases after eradication was not surprising. Further, we found an increase in FD, as recorded in the claims data, after eradication (Supplementary Figure 3), contrary to previous meta-analysis on RCTs reporting improvement or no change after eradication[45-47]. This discrepancy may be explained by our insurance claim system. For patients with dyspepsia symptoms and *H. pylori* infection, their diagnosis would be *H. pylori*-positive gastritis, which allows them to receive eradication therapy under insurance coverage. However, if they still had dyspeptic symptoms after successful eradication, their diagnosis

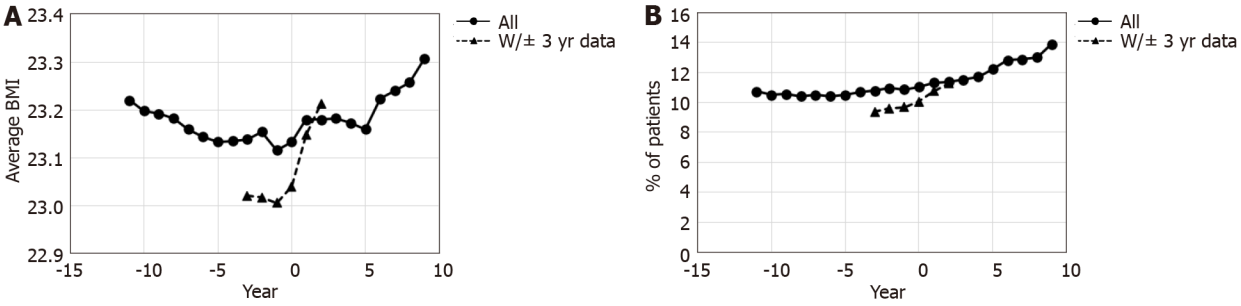

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Figure 6 Change in the average body mass index and percentage of patient with metabolic syndrome before and after the year of eradication. A: Average body mass index; B: Percentage of patients with metabolic syndrome. Year 0 is the year of primary eradication, and negative values represent years before primary eradication. BMI: Body mass index.

would be converted to FD, thereby increasing the number of patients with this label.

A protective effect of *H. pylori* infection against IBD has been suggested by an observational cohort study[48] and meta-analyses[19-21]. This relationship is explained by the effects of *H. pylori* infection on the immune system[18]. *H. pylori* infection increases gastric mucosal expression of a regulatory T cell marker, forkhead box P3, which decreases inflammatory Th1/Th17 responses and leads to suppression of inflammatory diseases including IBD[18,21]. Recently, metagenomic studies demonstrated an uneven recovery of the human gut microbiome after treatment with antibiotics[49,50], and the use of antibiotics was associated with an increased risk of developing both new-onset CD and UC. This risk was highest in the 1st year after antibiotic intake[51]. However, a higher incidence after eradication was also observed in all ages when comparing the 3-year period before and after eradication, and the incidence of CD and UC was higher in younger patients. In previous reports, eradication was not associated with an increased risk of IBD[52], or numerically, but not significantly increased[53]. Meanwhile, a significant increase in the risk of autoimmune diseases including IBD after eradication has also been reported[54]. A case-control study using Swedish national registry data showed that a history of antibiotic use is associated with IBD development, and that dispensation of multiple antibiotics increases the risk[55]. Our results are therefore consistent with the Swedish national registry data and should be investigated in other populations.

An inverse correlation of *H. pylori* infection with allergy has been reported in Japan[17] and other countries, and several mechanisms have been proposed[14-16]. *H. pylori* neutrophil-activating protein (HP-NAP), a virulence factor of *H. pylori*, reportedly stimulates neutrophils, increasing their production of oxygen radicals and adhesion to endothelial cells[56]. HP-NAP was shown to inhibit eosinophil infiltration and serum immunoglobulin E production in the lung, resulting in inhibition of interleukin (IL)-4 and IL-5 production in a mouse model[57]. These immunological changes in response to *H. pylori* eradication may be associated with the development of allergic diseases, as supported by our own findings. While the estimated number needed to treat for 3 years before and after eradication was calculated to be 58.8 for GU, 714.3 for DU, and 19.2 for gastritis and duodenitis, the estimated number needed to harm (NNH) for this period was found to be 12.3 for allergic diseases (unpublished data); this highlights the necessity of monitoring the effects of eradication therapy on allergy. Nevertheless, the effects of eradication therapy on chronic urticaria remain controversial; eradication therapy is recommended for those with chronic urticaria in Japanese guidelines[58]. Further large-scale prospective studies are warranted to understand the association between eradication and allergic diseases.

There are many reports on the relationship between *H. pylori* eradication and BMI and MS (Supplementary Table 4). Previous reports indicate that eradication generally increases BMI. An RCT conducted in South West England including *H. pylori*-infected patients with an average BMI of approximately 27 kg/m^2 reported an increase in BMI after eradication[59]. The increase in BMI after eradication is reportedly caused by the improvement of dyspepsia symptoms[59] and effects on the hormonal systems *via* ghrelin and leptin, which regulate food intake and appetite[24,59].

On the other hand, controversy remains about MS. Previous reports, including a Japanese study before 2010, have reported that dyslipidemia is exacerbated by eradication. However, recent reports have suggested that the factors that make up MS, especially dyslipidemia and diabetes, tend to improve after eradication (Supplementary Table 4). Recent Korean studies examining patients with an average BMI of approximately 24 kg/m^2 suggested an association between *H. pylori* infection and the risk of dyslipidemia[24,27], as well as a decrease in this risk after successful eradication[27]. The difference between the previous reports and our results may be due to the difference in the background of the population and the length of the observation period. Dyslipidemia is greatly affected not only by *H. pylori* status but also by age, sex, socioeconomic status, BMI, smoking status, diet, alcohol consumption, and exercise. Recently, changes in the gut microbiota after eradication and its effects on metabolism

have been discussed[26,60], and further elucidation of this pathophysiology is awaited.

Limits of the study

To our knowledge, this is the first study to investigate the effects of *H. pylori* eradication on a comprehensive disease spectrum in one of the largest target populations with a long observation period. However, this study has several limitations. First, we defined patients who received *H. pylori* eradication therapy based on prescription data. As such, there are no data about actual drug intake and the results of eradication success. However, we can assume that eradication rate is approximately 80% with PPI-based triple therapy, and 90% with the use of P-CAB-based triple therapy[61]. For those who could not achieve eradication, nearly 100% success can be achieved with a secondary regimen with metronidazole. Second, disease incidences were based on records of the diagnoses; thus, it was not possible to confirm the true onset of the development of the disease. In addition, some patients may have been diagnosed at the time of the medical visit or examination for eradication treatment, as suggested by the highest incidence of gastrointestinal diseases around the year of eradication. Consequently, the incidence might be overestimated around the year of eradication or later. Furthermore, it is possible that some diagnoses recorded in the claims based on drug prescriptions or diagnostic tests could be tentative or even false, although regular audit of the claims is done. In order to reduce such short-term influences, we also analyzed changes of disease incidence in a longer time-span. Third, although the NDB database is comprehensive, we only included a limited subset of patients when assessing the GERD status in patients with and without eradication, (only patients having health check-up data in the year of first GERD diagnosis were included) or when evaluating its effects on BMI and MS (only patients having health check-up data within 3 years before and after eradication were included). This affects the generalizability of these analyses. Finally, these findings may not be applicable to other countries, particularly those with different dietary habits and physical constitutions.

CONCLUSION

Our data obtained from a nationwide Japanese claims and health check-up database suggest an increased development of BE, IBD, allergic diseases, BMI, and MS, but not of GERD after *H. pylori* eradication. Although we observed a drastic decrease in the incidence of GU, DU, and gastritis, a large increase in allergic diseases may cancel out these beneficial effects, considering NNH. We believe that a comprehensive long-term assessment of the treatment effects, considering both favorable and unfavorable outcomes, is necessary for evaluating the true value of *H. pylori* eradication.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*) eradication therapy can prevent some diseases, including peptic ulcer disease and gastric cancer. However, potentially unfavorable effects of eradication therapy have also been reported for some diseases, such as gastroesophageal reflux disease (GERD), Barrett's esophagus (BE), inflammatory bowel disease (IBD), allergic diseases, and metabolic diseases. Consequently, both positive and negative impacts should be considered when assessing the effects of *H. pylori* eradication therapy.

Research motivation

This study compared the incidence of some diseases before and after *H. pylori* eradication therapy in order to assess the positive as well as negative effects of the therapy. The comprehensive evaluation of these effects in Japan, where large-scale eradication therapy was commenced earlier than it was in other countries because of coverage by universal healthcare, might provide useful information for clinicians worldwide.

Research objectives

The objective of this study was to compare the incidence of some diseases, which appear to be associated with *H. pylori* eradication therapy, before and after the eradication in order to obtain a comprehensive overview of the treatment effects.

Research methods

This study used a Japanese nationwide health claims database, the National Database of Health Insurance Claims and Specific Health Checkups of Japan (NDB; April 2009-March 2020), developed by the Japanese Ministry of Health, Labour and Welfare. The database contains almost all (≥ 95%) health insurance claims data issued in Japan as well as health check-up data for individuals who underwent

specific health check-ups. Patients with a prescription for primary *H. pylori* eradication therapy covered by national health insurance were analyzed as those who received primary *H. pylori* eradication therapy. The incidences of GERD, BE, other upper gastrointestinal diseases, and IBD; the prevalence of allergic diseases and metabolic syndrome (MS); and changes in body mass index (BMI) were examined before and after primary *H. pylori* eradication therapy.

Research results

In total, 5219731 patients who received primary eradication therapy were identified in the database. There was no significant increase in the incidence of GERD after eradication when considering the effects of aging and the reporting period. The incidence of BE was higher in the 3-year period after eradication than in the period before eradication for all age categories. The incidence of IBD and prevalence of allergic disease were also higher after eradication. In contrast, the incidences of gastric and DUs and gastritis were decreased after eradication. Among patients with at least one entry of health check-up data (1701111 patients), the percentage of patients with MS showed a slight increase following eradication (11.0% in the year of eradication and 12.2% after 5 years). Because this study only used information recorded in the claims database, the disease incidences were based on records of the diagnoses, and it was not possible to confirm the true onset of the development of the disease. The accuracy of the records of diagnoses also affected the results of this study.

Research conclusions

To our knowledge, this is the first study to examine the effects of *H. pylori* eradication therapy using a large-scale database in Japan. The results suggest that there is an increase in BMI and the development of BE, IBD, allergic diseases, and MS, but not in the development of GERD, after *H. pylori* eradication therapy. Although the treatment can drastically decrease the incidences of gastric and duodenal ulcers and gastritis, a considerable increase in allergic diseases may cancel out these beneficial effects.

Research perspectives

A comprehensive, long-term assessment of the treatment effects, with consideration of both favorable and unfavorable effects, is necessary for evaluating the true value of *H. pylori* eradication therapy.

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FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

Diagnostic and economic value of carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4 in gastrointestinal cancers

Hai-Ning Liu, Can Yao, Xiao-Fan Wang, Ning-Ping Zhang, Yan-Jie Chen, Dong Pan, Guo-Ping Zhao, Xi-Zhong Shen, Hao Wu, Tao-Tao Liu

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Abstract

BACKGROUND

The diagnostic and economic value of carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) and CA72-4 for gastrointestinal malignant tumors lacked evaluation in a larger scale.

AIM

To reassess the diagnostic and economic value of the three tumor biomarkers.

METHODS

A retrospective analysis of all 32857 subjects who underwent CEA, CA19-9, CA72-4, gastroscopy and colonoscopy from October 2006 to May 2018 was conducted. Then, we assessed the discrimination and clinical usefulness. Total cost, cost per capita and cost-effectiveness ratios were used to evaluate the economic value of two schemes (gastrointestinal endoscopy for all people without blood tests *vs* both gastroscopy and colonoscopy when blood tests were positive).

RESULTS

The analysis of 32857 subjects showed that CEA was a qualified biomarker for colorectal cancer (CRC), while the diagnostic efficiencies of CA72-4 were catastrophic for all gastrointestinal cancers (GICs). Regarding early diagnosis, only CEA could be used for early CRC. The combination of biomarkers didn't greatly increase the area under the curve. The economic indicators of CEA were superior to those of CA19-9, CA72-4 and any combination. At the threshold of 1.8 μg/L to 10.4 μg/L, all four indicators of CEA were lower than those in the scheme that conducted gastrointestinal endoscopy only. Subgroup analysis implied that the health checkup of CEA for people above 65 years old was economically valuable.

CONCLUSION

CEA had qualified diagnostic value for CRC and superior economic value for GICs, especially for elderly health checkup subjects. CA72-4 was not suitable as a diagnostic biomarker.

Key Words: Diagnostic test; Economic analysis; Cost-effectiveness analysis; Decision curve analysis

Core Tip: This is a retrospective study to reassess the diagnostic and economic value of traditional tumor biomarkers carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) and CA72-4 for gastrointestinal malignant tumors in a large sample with novel indicators. Instead of increasing the diagnostic value, CA72-4 should be removed from the list of the health checkup items to avoid the waste of social medical resources for CEA were superior to those of CA19-9, CA72-4 or any other combinations in which it could be applied for early colorectal cancer and a health checkup of CEA for people above 65 years old was economically valuable.

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INTRODUCTION

Blood carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are widely used as classic diagnostic markers for malignant tumors, and they are recommended by several clinical guidelines for gastrointestinal cancer (GIC) screening[1-3]. Following the introduction of the CEA and CA19-9 assessment, in 1990, blood CA72-4 was proposed as a diagnostic biomarker for gastric cancer (GC)[4]. Subsequent studies showed that CA72-4 could be used to diagnose GC and colorectal cancer (CRC)[5,6]. These studies reported sensitivities of 19%-47% in GC and 25%-43% in CRC at the cut-off value of 6 kU/L[7-13]. The clinical guidelines published by the European Group on Tumor Markers (EGTM) in 2003 suggested that CA72-4 could be a potential biomarker for CRC[14].

Based on these previous studies, blood CA72-4 began to be widely used as a tumor biomarker since 2010 in China. Nevertheless, after large-scale clinical application, we noticed, empirically, an extremely high false positive rate of CA72-4 for diagnosis. A positive result would lead the subject to undergo further examinations, including gastroscopy, colonoscopy, chest computed tomography (CT), abdominal CT, and even positron emission tomography-CT (PET-CT). The blood test with a low positive predictive value (PPV) not only brings unnecessary anxiety, invasive examinations, and extra costs to the subjects but also leads to the waste of medical resources and increases the social medical burden.

The massive data and real-world diagnostic cohorts make it possible to further explore the diagnostic and economic value of biomarkers. Through a real-world diagnostic cohort, we comprehensively analyzed the differences in the levels of CEA, CA19-9, and CA72-4 and their diagnostic and economic value in gastrointestinal tumors. Four indicators were used to comprehensively evaluate the economic value, namely, the total cost and the average cost per person for each positive patient diagnosed and their corresponding cost-effectiveness ratios. We evaluated whether age and health checkup could help us make useful recommendations for thresholds of tumor biomarkers and medical insurance policies.

Table 1 The six schemes and examination prices in economic analysis

Item	Description
Schemes	Scheme 1 Gastrointestinal endoscopy for all people without blood tests
	Scheme 2 Both of gastroscopy and colonoscopy when blood tests were positive
	Scheme 3 Gastroscopy first when blood tests were positive, and then colonoscopy when the result of gastroscopy was negative
	Scheme 4 Colonoscopy first when blood tests were positive, and then gastroscopy when the result of colonoscopy was negative
	Scheme 5 Only gastroscopy when blood tests were positive
	Scheme 6 Only colonoscopy when blood tests were positive
Examination prices	CEA, $4.64; CA19-9, $7.25; CA72-4, $7.25
	Gastroscopy & biopsy, $87.99
	Colonoscopy, $57.98; biopsy after colonoscopy, $32.62

The prices of gastroscopy and colonoscopy did not include that of intravenous anesthesia. The costs of all examinations were from Zhongshan Hospital in 2019. All costs were converted to United States dollars. CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4.

MATERIALS AND METHODS

Study population

We retrospectively analyzed all patients from October 2006 to May 2018. The inclusion criteria included: (1) Patients from the medical examination center, outpatient department or inpatient department of Zhongshan Hospital of Fudan University; and (2) patients had completed all five examinations, namely, CEA, CA19-9, CA72-4, gastroscopy and colonoscopy, within half a year. The exclusion criteria were as follows: (1) Duplicate patients; and (2) patients who had accepted anti-tumor therapies such as radiotherapy, chemotherapy or surgery.

Data extraction

All data were abstracted from our hospital information system (HIS). They included general information (*e.g.*, age, sex, medical record number, whether health checkup, past history), the concentrations of each of the three tumor biomarkers, reports of auxiliary examinations (*e.g.*, endoscopy, pathology, ultrasonography, CT, magnetic resonance, PET-CT, electrocardiogram), and the medical records of outpatients and inpatients. The generation time of these data was also provided.

The concentrations of serum CEA, CA19-9, and CA72-4 were measured with an electrochemiluminescence immunoassay (Elecsys2010, Roche Diagnostics, indianapolis, IN, United States). The traditional cut-off values for CEA, CA19-9, and CA72-4 were 5 μg/L, 37 kU/L, and 6 kU/L, respectively.

According to the regular practice of our hospital, pathological biopsy was taken when gastroscopy was performed, while colon biopsy was not necessary taken unless some lesions were found by colonoscopy. The diagnosis of GIC depends on the gold standard of pathology, and other gastrointestinal diseases are diagnosed by endoscopy and pathology. Other malignant tumors were comprehensively judged based on the medical history, pathology and imaging exams that we could collect. TNM staging of cancers was based on the American Joint Committee on Cancer Staging or case data at that time.

Economic analysis

According to the type of test and the order of endoscopy procedures, we assumed six schemes (Table 1). Four economic indicators combined with the proportion of endoscopies and the missed diagnosis rate was used to evaluate the economic value of tumor biomarkers. The four economic indicators were the total cost and cost per capita of correctly diagnosing one case of GIC and the cost-effectiveness ratio of the above two indicators. The cost-effectiveness ratio was the total cost or cost per capita divided by sensitivity. We assumed that the missed diagnosis rate and misdiagnosis of endoscopy plus necessary pathological examination for gastrointestinal malignancies were all 0.

The costs of blood tests, endoscopy and pathological examination were the cost of these procedures at Zhongshan Hospital in 2019 (Table 1). All costs were converted to United States dollars.

Considering the preliminary results, further analyses were performed on Scheme 1 (gastrointestinal endoscopy for all people without blood tests) and Scheme 2 (both gastroscopy and colonoscopy when blood tests were positive). We also calculated 9 conditions when CEA and CA19-9 were combined. They

Table 2 The clinical characteristics of subjects with and without gastric cancer, colorectal cancer and gastrointestinal cancers

	Age median (quartile)	*P* value	Male, *n* (%)	Female, *n* (%)	*P* value
Gastric cancer	61 (51, 68)	< 0.001	268 (68.4)	124 (31.6)	0.084
Non-gastric cancer	48 (42, 56)		20831 (64.2)	11634 (35.8)	
Colorectal cancer	62 (55, 70)	< 0.001	522 (58.5)	370 (41.5)	< 0.001
Non-colorectal cancer	48 (42, 55)		20577 (64.4)	11388 (35.6)	
Gastrointestinal cancer	62 (53, 69)	< 0.001	816 (62.4)	491 (37.6)	0.170
Non-gastrointestinal cancer	48 (42, 55)		20283 (64.3)	11267 (35.7)	

The *P* value was calculated with the Wilcoxon test for age and chi-square test for sex. The bold font indicates that the *P* value was less than 0.05.

were parallel (any positive was considered positive), serial (all positive was considered positive), and the formula under the traditional cut-off value (the coefficients of CEA and CA19-9 were calculated according to the logistic regression), the minimum total cost, and the minimum total cost-effectiveness ratio.

Subgroup analysis (age, health checkup/active consultation) was utilized to analyze the economic value of the three biomarkers under the traditional threshold, with a view to drawing some medical insurance recommendations.

Statistical analysis

The statistical analyses were performed using R software 3.3.5 (R Foundation for Statistical Computing, Vienna, Austria). The level of significance was set at $P < 0.05$. All tests were two-sided.

Student's *t*-test or Wilcoxon test was used to assess the differences in continuous variables, as appropriate. The chi-square test was used for counting variables. Correlations between two variables were calculated by Pearson correlation analysis or Spearman correlation analysis. The influences of age and sex on the biomarker levels were analyzed with the regression coefficient of linear regression. Categorical regression analysis was utilized to calculate the regression coefficient quantification of each stage of GC and CRC.

The diagnostic value was evaluated by means of the area under the curve (AUC) values of the receiver operating characteristics (ROC) curve, as well as the diagnostic odds ratio (DOR), sensitivity, specificity, Youden index (sensitivity + specificity-1), accuracy, predicted value and likelihood ratio on the traditional and best cut-off values. The best cut-off value referred to the threshold when the Youden index was the largest. When multiple diagnostic biomarkers were combined, logistic regression was used to calculate the formula coefficients. We used Delong's test to compare AUC.

Decision curve analysis (DCA) was performed to determine the clinical usefulness of the radiomics nomogram by quantifying the net benefits at different threshold probabilities. The clinical net benefit was defined as the true positive rate (sensitivity) minus the false positive rate (misdiagnosis rate) and was then weighted by the relative damage of the positive rate and the negative rate.

RESULTS

Clinical characteristics

According to the inclusion criteria, we screened a total of 32857 subjects aged 15 to 97 years in the HIS, including 21099 males and 11758 females. There were 24045 subjects who underwent health checkup and 8812 subjects with an active consultation (Figure 1). The ages and sexes of the subjects with GC, CRC, and GIC were significantly different from those of the subjects without the disease (Table 2).

The constituent ratios of the diseases detected by gastroscopy, colonoscopy and pathological examination are displayed in Table 3. Among them, there were 392 GC cases, 892 CRC cases and 1307 GIC cases.

Serum levels of tumor biomarkers

The concentrations of the three biomarkers were skewed (Figure 2). The correlations between the pairwise biomarkers are shown in Table 4. We found that there were significant correlations between CEA and CA19-9 in all subjects and various GICs, and the correlation coefficients were all exceed 0.245.

The median values for CEA, CA19-9, and CA72-4 Level were 1.67 μg/L, 8.50 kU/L and 1.60 kU/L, respectively. The expression levels of the biomarkers for the diseases that had more than 30 cases are shown in Table 5. The concentrations of the three biomarkers in patients with several malignant tumors were significantly different from those without malignant tumors (Figure 3). The CEA level increased

Table 3 The number and proportions of diseases in the gastroscopy, colonoscopy and pathological examination groups

Examination	Disease	No.	%
Gastroscope (without pathological examination)	Esophagitis	2887	8.8%
	Esophageal erosion	88	0.3%
	Esophageal ulcer	30	0.1%
	Esophageal protuberant lesion	491	1.5%
	Esophageal non-protuberant lesion	77	0.2%
	Barrett's esophagus	66	0.2%
	Bile reflux	1344	4.1%
	Gastric atrophy	45	0.1%
	Gastric erosion	12457	37.9%
	Gastric hemorrhage	1117	3.4%
	Gastric ulcer	1088	3.3%
	Gastric protuberant lesion	3329	10.1%
	Gastric non-protuberant lesion	225	0.7%
	Duodenitis	1473	4.5%
	Duodenal erosion	26	0.1%
	Duodenal ulcer	1548	4.7%
	Duodenal protuberant lesion	666	2.0%
	Duodenal non-protuberant lesion	31	0.1%
Colonoscopy (without pathological examination)	Colorectitis	653	2.0%
	Colorectal erosion	29	0.1%
	Colorectal ulcer	99	0.3%
	Colorectal protuberant lesion	7312	22.3%
	Colorectal non-protuberant lesion	36	0.1%
Pathological examination	Esophageal mucositis	398	1.2%
	Esophageal dysplasia	44	0.1%
	Esophageal adenoma	1	< 0.1%
	Esophageal hyperplastic polyp	2	< 0.1%
	Esophageal glandular hyperplasia	4	< 0.1%
	Chronic atrophic gastritis	1809	5.5%
	Gastric dysplasia	308	0.9%
	Gastric adenoma	13	< 0.1%
	Gastric hyperplastic polyps	117	0.4%
	Gastric glandular hyperplasia	761	2.3%
	Gastric juvenile polyps	1	< 0.1%
	Duodenal mucositis	276	0.8%
	Duodenal dysplasia	24	0.1%
	Duodenal adenoma	12	< 0.1%
	Duodenal hyperplastic polyps	10	< 0.1%
	Duodenal gland hyperplasia	31	0.1%
	Colorectal mucositis	2206	6.7%
	Colorectal high-grade intraepithelial neoplasia	330	1.0%

	Colorectal low-grade intraepithelial neoplasia	3364	10.2%
	Colorectal adenoma	3707	11.3%
	Colorectal hyperplastic polyps	1037	3.2%
	Colorectal inflammatory polyps	13	< 0.1%
	Colorectal gland hyperplasia	567	1.7%
	Colorectal juvenile polyps	3	< 0.1%
	Peutz-Jeghers polyps	5	< 0.1%
	Familial polyposis coli	3	< 0.1%
	Esophageal cancer	57	0.2%
	Gastric cancer	392	1.2%
	Duodenal cancer	25	0.1%
	Small intestine cancer	4	< 0.1%
	Colorectal cancer	892	2.7%
	Liver cancer	127	0.4%
	Pancreatic cancer	47	0.1%
	Gallbladder cancer	20	0.1%
	Bile duct cancer & ampulla cancer	10	< 0.1%
	Lung cancer	129	0.4%
	Breast cancer	55	0.2%
	Ovarian cancer	57	0.2%
	Uterine malignancy	28	0.1%
	Kidney cancer	37	0.1%
	Prostate cancer	28	0.1%
	Bladder Cancer	17	0.1%
	Leukemia	1	< 0.1%
	Lymphoma	29	0.1%
	Other malignant tumors	15	< 0.1%

Table 4 Correlation analysis of biomarker levels

	Correlation coefficient			*P* value		
	CEA and CA19-9	CEA and CA72-4	CA19-9 and CA724-4	CEA and CA19-9	CEA and CA72-4	CA19-9 and CA724-4
Whole	0.245	-0.005	-0.046	< 0.001	0.359	< 0.001
Gastric cancer	0.291	0.048	0.022	< 0.001	0.342	0.657
Colorectal cancer	0.385	0.2	0.169	< 0.001	< 0.001	< 0.001
Gastrointestinal cancer	0.354	0.164	0.134	< 0.001	< 0.001	< 0.001

P values and correlation coefficients were calculated with Pearson correlation analysis or Spearman correlation analysis. The bold font indicates that the *P* value was less than 0.05. CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4.

but did not exceed 0.3 μg/L in esophageal erosion, gastric erosion, gastric ulcer, chronic atrophic gastritis, and colorectal adenoma.

The influences of age and sex on the biomarker levels are presented in Table 6. Due to the fact that the patients with malignant tumors were elder, the age baselines of the patients with and without tumors were no equal. Moreover, the sex baseline of the CRC patients was not the same. The correlation coefficients of age and sex were both less than 0.25, indicating small influences. The regression coefficients

Table 5 The biomarker levels, comparisons between subjects with and without diseases and area under the curves of carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4

Disease	No.	CEA			CA19-9			CA72-4		
		Median (quartile) (μg/L)	*P* value	AUC	Median (quartile) (kU/L)	*P* value	AUC	Median (quartile) (kU/L)	*P* value	AUC
Whole	32857	1.67 (1.13, 2.41)	-	-	8.50 (5.60, 13.50)	-	-	1.60 (1.05, 3.20)	-	-
Esophagitis	3137	1.88 (1.29, 2.72)	< 0.001	0.566	8.60 (5.70, 13.50)	0.439	0.504	1.60 (1.10, 3.40)	0.001	0.518
Esophageal erosion	109	1.84 (1.40, 2.90)	0.007	0.575	8.30 (5.00, 15.00)	0.938	0.498	1.50 (1.10, 3.20)	0.644	0.487
Esophageal ulcer	30	1.73 (0.90, 2.29)	0.818	0.488	7.90 (5.73, 10.38)	0.166	0.573	1.59 (1.10, 3.95)	0.572	0.470
Barrett's esophagus	66	1.91 (1.21, 3.01)	0.095	0.559	8.95 (6.25, 12.70)	0.565	0.520	1.95 (1.10, 3.98)	0.306	0.536
Bile reflux	1344	1.65 (1.07, 2.43)	0.435	0.506	8.90 (5.60, 14.73)	0.121	0.512	1.60 (1.10, 3.30)	0.130	0.512
Gastric erosion	13094	1.78 (1.22, 2.57)	< 0.001	0.555	8.60 (5.70, 13.70)	0.006	0.509	1.60 (1.00, 3.20)	0.748	0.499
Gastric ulcer	1091	1.98 (1.41, 2.98)	< 0.001	0.598	8.40 (5.50, 14.40)	0.646	0.496	1.60 (1.00, 3.20)	0.789	0.498
Gastric hemorrhage	1125	1.68 (1.12, 2.46)	0.492	0.506	8.70 (5.80, 14.50)	0.092	0.515	1.60 (1.10, 3.40)	0.331	0.509
Chronic atrophic gastritis	1839	1.90 (1.32, 2.89)	< 0.001	0.578	9.20 (6.00, 14.90)	< 0.001	0.535	1.70 (1.10, 3.40)	< 0.001	0.528
Gastric xanthoma	100	1.65 (1.13, 2.41)	0.935	0.498	10.35 (6.45, 15.18)	0.072	0.552	1.80 (1.20, 3.43)	0.111	0.546
Gastrointestinal stromal tumor	48	1.56 (1.09, 2.69)	0.903	0.505	7.80 (5.78, 13.41)	0.614	0.521	1.50 (1.10, 2.23)	0.683	0.517
Gastric hyperplastic polyps	117	1.71 (1.14, 2.89)	0.282	0.529	11.20 (7.00, 22.72)	< 0.001	0.612	1.70 (1.10, 2.40)	0.964	0.501
Gastric glandular hyperplasia	761	1.57 (1.09, 2.29)	0.050	0.521	9.50 (6.00, 15.00)	< 0.001	0.543	1.70 (1.10, 3.80)	0.010	0.527
Colorectitis	2592	1.83 (1.25, 2.70)	< 0.001	0.552	8.70 (5.70, 14.30)	0.014	0.515	1.60 (1.10, 3.40)	0.034	0.513
Colorectal erosion	167	1.72 (1.17, 2.73)	0.198	0.529	8.80 (5.95, 13.90)	0.495	0.515	1.60 (1.00, 3.10)	0.788	0.494
Colorectal ulcer	107	1.54 (1.03, 2.61)	0.658	0.512	9.10 (6.30, 16.90)	0.069	0.551	1.70 (1.09, 2.80)	0.859	0.495
Colorectal hemorrhage	36	1.57 (1.12, 3.14)	0.799	0.488	9.05 (5.73, 13.75)	0.550	0.529	1.25 (0.90, 1.95)	0.061	0.590
Colorectal cyst	41	1.53 (0.93, 2.70)	0.455	0.534	9.20 (6.50, 13.70)	0.321	0.545	1.40 (1.10, 2.40)	0.353	0.542
Colorectal adenoma	3707	1.91 (1.29, 2.84)	< 0.001	0.578	9.04 (6.00, 14.70)	< 0.001	0.532	1.60 (1.10, 3.30)	0.010	0.513
Colorectal hyperplastic polyps	1037	1.88 (1.31, 2.75)	< 0.001	0.565	8.70 (5.90, 13.70)	0.065	0.517	1.60 (1.00, 3.20)	0.379	0.492
Colorectal gland hyperplasia	567	1.87 (1.35, 2.62)	< 0.001	0.560	8.30 (5.45, 13.45)	0.308	0.512	1.50 (1.10, 3.30)	0.687	0.505
Esophageal cancer	57	2.34 (1.30, 3.78)	< 0.001	0.645	9.40 (6.40, 20.00)	0.114	0.560	2.00 (1.20, 4.20)	0.073	0.568
Gastric cancer	392	2.15 (1.35, 4.13)	< 0.001	0.625	10.30 (5.70, 20.23)	< 0.001	0.577	2.00 (1.10, 5.70)	< 0.001	0.570
Colorectal cancer	892	3.25 (1.78, 11.55)	< 0.001	0.736	13.30 (7.10, 33.45)	< 0.001	0.649	2.30 (1.20, 5.90)	< 0.001	0.598
Liver cancer	127	4.27 (2.15, 7.46)	< 0.001	0.786	17.30 (7.40, 39.15)	< 0.001	0.674	1.60 (1.15, 3.75)	0.070	0.547
Pancreatic cancer	47	3.20 (1.98, 9.63)	< 0.001	0.771	99.60 (16.95, 307.15)	< 0.001	0.830	3.10 (1.35, 9.60)	< 0.001	0.680
Lung cancer	129	4.25 (2.15, 16.67)	< 0.001	0.787	12.10 (8.20, 25.50)	< 0.001	0.668	3.40 (1.40, 9.00)	< 0.001	0.660
Breast cancer	55	2.39 (1.44, 6.06)	< 0.001	0.654	15.60 (8.30, 27.20)	< 0.001	0.693	2.10 (1.20, 4.70)	0.022	0.589
Ovarian cancer	57	1.78 (1.08, 5.63)	0.228	0.546	21.85 (8.50, 130.50)	< 0.001	0.718	6.40 (1.50, 17.70)	< 0.001	0.698

Thyroid cancer	74	1.71 (1.06, 2.48)	0.936	0.497	9.90 (6.80, 15.28)	0.067	0.562	1.70 (1.03, 3.08)	0.874	0.495
Kidney cancer	37	2.37 (1.16, 3.70)	0.015	0.615	10.60 (7.70, 17.46)	0.039	0.598	2.20 (1.40, 5.20)	0.050	0.593
Malignant tumors (except thyroid cancer)	1955	2.65 (1.49, 6.70)	< 0.001	0.692	12.00 (6.80, 28.30)	< 0.001	0.636	2.20 (1.20, 5.90)	< 0.001	0.589

The bold font of the *P* value indicates that the *P* value was less than 0.05 in the Wilcoxon test. The bold font of the area under the curve (AUC) indicates that the AUC value was greater than 0.7. CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4; AUC: Area under the curve.

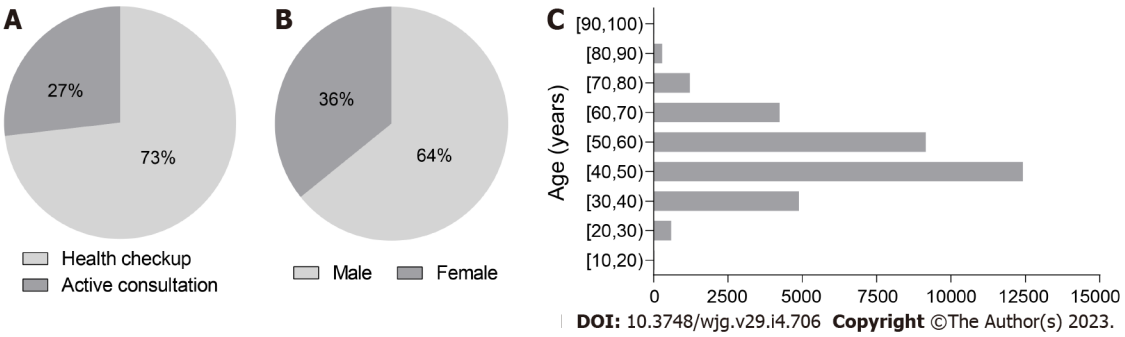

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Figure 1 Fan charts and bar plots of the clinical characteristic. A-C: There were 24045 subjects who underwent health checkup and 8812 subjects with an active consultation.

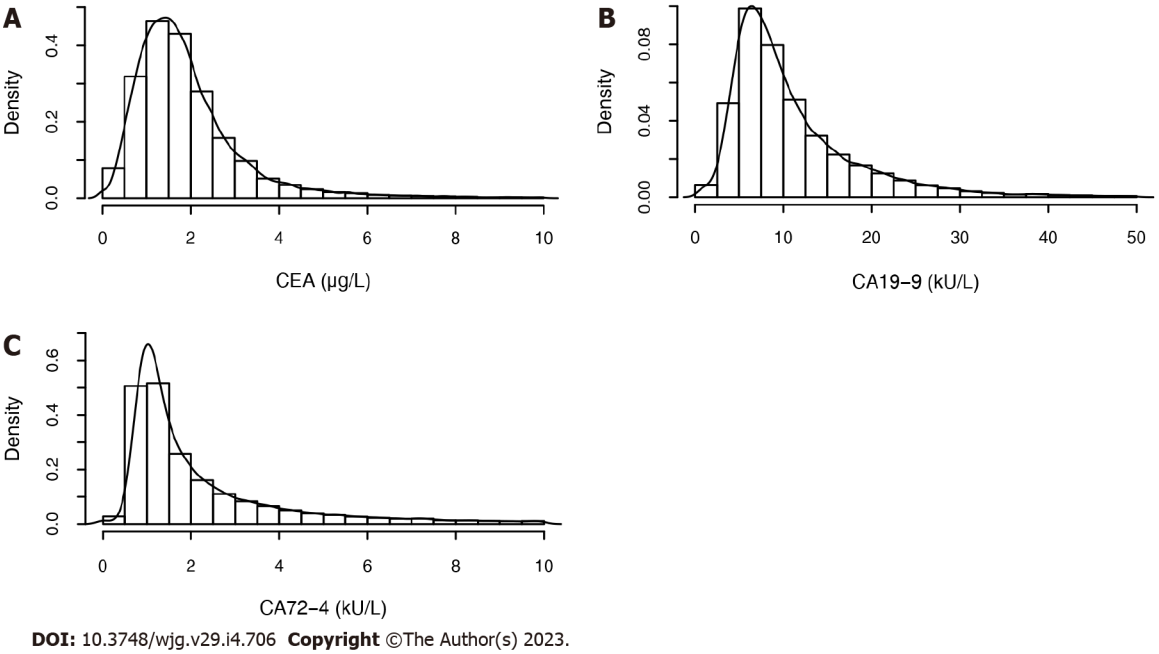

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Figure 2 Histograms of carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4. A: Carcinoembryonic antigen; B: Carbohydrate antigen 19-9; C: Carbohydrate antigen 72-4. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 72-4: Carbohydrate antigen 72-4.

were used to calculate the effect of age on the biomarker levels. CEA, CA19-9, and CA72-4 increased by 0.41, 2.69, and 0.69, respectively, for the subjects without malignant tumors for every 10-year increase.

The biomarker levels in different malignant tumor stages are shown in Table 7 and Figure 4.

Diagnostic accuracies of tumor biomarkers

The AUCs of the three biomarkers in various benign and malignant diseases are displayed in Table 5, and the ROC curves are shown in Figure 5. An AUC above 0.7 was of moderate diagnostic value, and an AUC above 0.9 was of high diagnostic value. We found that even though the biomarker levels of several

Table 6 Correlation analysis and linear regression analysis of biomarker levels and clinical characteristics

		Age			Gender		
		***P* value**	**Correlation coefficient**	**Regression coefficient**	***P* value**	**Correlation coefficient**	**Regression coefficient**
CEA	Whole	< 0.001	0.227	0.176	< 0.001	0.236	0.004
	With malignant tumors	< 0.001	0.231	0.263	< 0.001	0.144	-2.965
	Without malignant tumors	< 0.001	0.195	0.041	< 0.001	0.248	0.472
CA19-9	Whole	< 0.001	0.135	1.076	< 0.001	-0.070	-1.400
	With malignant tumors	< 0.001	0.111	1.898	0.356	-0.021	-
	Without malignant tumors	< 0.001	0.113	0.269	< 0.001	-0.071	-2.482
CA72-4	Whole	< 0.001	0.084	0.076	< 0.001	-0.043	-0.927
	With malignant tumors	0.064	0.042	-	0.814	-0.005	-
	Without malignant tumors	< 0.001	0.069	0.052	< 0.001	-0.044	-0.773

The bold font indicates that the *P* value was less than 0.05. CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4.

diseases were significantly different, the diagnostic values of these biomarkers were not high enough. The AUC of the CEA level reached 0.7 for CRC, liver cancer, pancreatic cancer and lung cancer, while those of the CA19-9 Level reached 0.830 for pancreatic cancer and 0.7 for ovarian cancer. There was no disease in which the AUC of CA72-4 reached 0.7.

We show the diagnostic value of GC, CRC and gastrointestinal malignant tumors (the DOR, sensitivity, specificity, Youden index, accuracy, predictive value, likelihood ratio under the traditional and the best threshold) in Table 8. Furthermore, we provide several criteria for evaluating their diagnostic efficiencies as the qualified standards: positive likelihood ratio, negative likelihood ratio and DOR should be > 5.0, < 0.2 and > 10.0, respectively. Generally, there is no ideal biomarker for GC. In this study, CEA was better than CA19-9 and CA72-4. The positive likelihood ratio and DOR of CEA and CA19-9 were qualified for CRC and GIC, while those of CA72-4 were not qualified for GC, CRC or GIC.

The AUCs of diverse subgroups, including age, health checkup/active consultation and malignant tumor stage, are shown in Table 9. We defined an AUC greater than 0.7 as the qualified line. Then, the AUCs of CEA, CA19-9, and CA72-4 in the health checkup population were all unqualified. If we looked at the stages alone, CEA for stage-IV GC, CA19-9 for stage-IV CRC and CEA for stage-II-IV CRC were qualified. However, neither CEA nor CA199 can diagnose early GICs.

The DCA curves of the three biomarkers are presented in Figure 6. The DCA curve showed that under the traditional threshold and the best threshold, the clinical benefits of CEA were higher than those of CA19-9, while the clinical benefits of CA72-4 were the lowest.

Four panels were conducted with the combination of the three biomarkers. We selected the panel with the highest AUC and compared it with the single biomarker with the highest AUC (Table 10). The combination of biomarkers in the CRC and gastrointestinal malignant tumors significantly increased the AUC (Delong's test, P < 0.05) by less than 0.3, while that in GC did not. Therefore, the combination of the three biomarkers could not greatly improve the diagnostic value.

Economic analysis of tumor biomarkers with endoscopies

We analyzed the four economic indicators of the six schemes with changes in the serum levels of the three biomarkers, as shown in Figure 7. For gastroscopy only, the total cost and cost-effectiveness ratio of correctly diagnosing one case of GIC were unacceptably high. For colonoscopy only, various cost indicators were reduced within a certain range of biomarker levels. The four economic indicators of CEA in Scheme 6 (only colonoscopy conducted when blood tests were positive) were lower than those in other schemes because the diagnostic efficiencies of CEA for CRC were high, and the prevalence rate of CRC was higher than that of GC in this study. If both gastroscopy and colonoscopy were conducted, the influence of the order of gastroscopy on the four economic indicators was small. Therefore, in the follow-up study, we only calculated the economic indicators in Scheme 2 (both gastroscopy and colonoscopy when blood tests were positive) compared to those in Scheme 1 (both gastroscopy and colonoscopy for all people without blood tests).

Table 7 The biomarker levels and categorical regression analysis of each stage of gastric cancer and colorectal cancer

		Gastric cancer		Colorectal cancer	
		Median (quartile)	Quatization	Median (quartile)	Quatization
CEA (μg/L)	CIS	1.74 (1.45, 2.18)	-0.983	2.04 (1.17, 2.32)	-1.695
	Stage I	1.78 (1.29, 2.79)	-0.983	2.30 (1.45, 4.10)	-1.252
	Stage II	2.16 (1.07, 4.03)	-0.835	3.42 (2.13, 9.26)	-0.680
	Stage III	2.37 (1.31, 5.78)	-0.176	3.28 (2.00, 8.21)	-0.680
	Stage IV	3.79 (1.76, 29.1)	1.346	10.1 (2.57, 57.4)	1.168
CA19-9 (kU/L)	CIS	8.60 (5.05, 12.7)	-1.138	8.66 (6.18, 13.2)	-0.963
	Stage I	9.25 (5.88, 14.4)	-1.138	9.50 (6.20, 14.1)	-0.963
	Stage II	7.73 (5.53, 16.8)	-1.138	12.4 (7.38, 28.8)	-0.812
	Stage III	12.2 (5.63, 37.7)	0.842	13.1 (7.33, 23.0)	-0.790
	Stage IV	11.8 (5.08, 28.7)	0.903	28.9 (10.5, 216.4)	1.192
CA72-4 (kU/L)	CIS	1.50 (0.90, 3.50)	-1.203	1.50 (1.00, 2.03)	-1.550
	Stage I	1.80 (1.20, 4.33)	-0.977	1.70 (1.10, 3.20)	-1.060
	Stage II	1.90 (1.18, 4.30)	-0.789	2.10 (1.20, 3.81)	-0.818
	Stage III	2.10 (1.30, 4.63)	-0.164	1.95 (1.10, 4.21)	-0.679
	Stage IV	4.10 (1.00, 12.0)	1.340	4.75 (1.50, 15.2)	1.182

CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4; CIS: Carcinoma *in situ*.

Table 8 Diagnostic efficiencies of gastric cancer, colorectal cancer and gastrointestinal cancers at the traditional and best cut-off values

		Gastric cancer			Colorectal cancer			Gastrointestinal cancers		
		CEA	CA19-9	CA72-4	CEA	CA19-9	CA72-4	CEA	CA19-9	CA72-4
	AUC	0.625	0.577	0.570	0.736	0.649	0.598	0.705	0.627	0.590
Traditional cut-off value	Cut-off value	5.0	37.0	6.0	5.0	37.0	6.0	5.0	37.0	6.0
	DOR	6.083	5.089	2.220	14.854	11.895	2.376	12.459	10.367	2.337
	Sensitivity	0.227	0.140	0.240	0.377	0.241	0.249	0.323	0.210	0.243
	Specificity	0.954	0.969	0.876	0.961	0.974	0.878	0.963	0.975	0.879
	Youden index	0.181	0.109	0.115	0.338	0.215	0.127	0.286	0.185	0.122
	Accuracy	0.945	0.959	0.868	0.945	0.954	0.861	0.938	0.945	0.854
	PPV	0.056	0.052	0.023	0.212	0.204	0.054	0.266	0.261	0.077
	NPV	0.990	0.989	0.990	0.982	0.979	0.977	0.972	0.968	0.966
	PLR	4.927	4.516	1.927	9.640	9.269	2.034	8.759	8.400	2.012
	NLR	0.810	0.888	0.868	0.649	0.779	0.856	0.703	0.810	0.861
Best cut-off value	Cut-off value	2.6	16.3	3.8	2.8	20.7	2.0	2.5	19.6	3.4
	DOR	2.687	2.233	2.038	6.345	4.825	1.933	4.419	3.872	2.068
	Sensitivity	0.423	0.324	0.349	0.558	0.361	0.566	0.556	0.339	0.375
	Specificity	0.785	0.823	0.791	0.834	0.895	0.597	0.779	0.883	0.775
	Youden index	0.209	0.147	0.141	0.392	0.256	0.163	0.335	0.222	0.150
	Accuracy	0.781	0.817	0.786	0.827	0.881	0.596	0.770	0.861	0.759
	PPV	0.023	0.022	0.020	0.086	0.088	0.038	0.094	0.107	0.065

	NPV	0.991	0.990	0.990	0.985	0.980	0.980	0.977	0.970	0.968
	PLR	1.972	1.833	1.675	3.363	3.445	1.405	2.519	2.900	1.667
	NLR	0.734	0.821	0.822	0.530	0.714	0.727	0.570	0.749	0.806

The best cut-off value referred to the threshold when the Youden index was the largest. CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4; AUC: Area under the curve; DOR: Diagnostic odds ratio; PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio; NLR: Negative likelihood ratio.

Table 9 Subgroup analysis of area under the curve for gastric cancer, colorectal cancer and gastrointestinal cancers

	Gastric cancer			Colorectal cancer			Gastrointestinal cancer		
	CEA	CA19-9	CA72-4	CEA	CA19-9	CA72-4	CEA	CA19-9	CA72-4
Whole	0.625	0.577	0.570	0.736	0.649	0.598	0.705	0.627	0.590
≥ 60 years	0.585	0.521	0.544	0.701	0.614	0.577	0.675	0.592	0.572
< 60 years	0.578	0.571	0.570	0.683	0.616	0.593	0.648	0.598	0.583
HC	0.570	0.570	0.525	0.584	0.539	0.514	0.584	0.554	0.526
AC	0.595	0.544	0.547	0.724	0.637	0.577	0.696	0.615	0.571
CIS	0.551	0.478	0.542	0.540	0.536	0.526	-	-	-
Stage I	0.554	0.525	0.565	0.675	0.546	0.512	-	-	-
Stage II	0.603	0.489	0.591	0.781	0.657	0.578	-	-	-
Stage III	0.658	0.645	0.603	0.770	0.642	0.565	-	-	-
Stage IV	0.739	0.614	0.634	0.810	0.778	0.698	-	-	-

The bold font indicates that the area under the curve value was more than 0.7. CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4; HC: Health checkup; AC: Active consultation; CIS: Carcinoma *in situ*.

Table 10 The best single biomarker and the best combination of biomarkers for gastric cancer, colorectal cancer and gastrointestinal cancers

	Best combination		Best single biomarker		*P* value
	Biomarkers	AUC	Biomarker	AUC	
Gastric cancer	CEA + CA19-9 + CA72-4	0.653	CEA	0.625	0.067
Colorectal cancer	CEA + CA19-9	0.761	CEA	0.736	< 0.001
Gastrointestinal cancers	CEA + CA19-9	0.727	CEA	0.705	< 0.001

The bold font indicates that the *P* value was less than 0.05 in Delong's test. AUC: Area under the curve; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4.

In terms of threshold selection, we found that the traditional threshold of CEA (5 μg/L) was exactly between the CEA level under the minimum total cost-effectiveness ratio (4.3 μg/L) and that under the minimum total cost (equal to cost-effectiveness ratio per capita, 8.7 μg/L). If we decrease the cut-off value, the four indicators grew rapidly. If we increase the cut-off value, then the total cost-effectiveness ratio rose sharply, while the other three indicators had fewer changes. One can use 5 μg/L for CEA as an economic cut-off value. For CA19-9, we found that a similarly high economic cut-off value was approximately 30 kU/L, not the traditional threshold of 37 kU/L. Compared with that at the threshold of 30 kU/L, the total cost-effectiveness ratio at the threshold of 37 kU/L was greatly increased because of the lower sensitivity of the marker. We evaluated the economic efficiencies as the qualified standard: all four indicators in Scheme 2 were lower than those in Scheme 1. CEA met the standards at the threshold of 1.8 μg/L to 10.4 μg/L. CA19-9 and CA72-4 failed at the whole threshold, caused by the high total cost-effectiveness ratio in Scheme 2.

Compared with CEA, the combination of the three biomarkers in pairs or altogether caused the cost and cost-effectiveness ratio to be higher (Table 11). From an economic perspective, the combination of

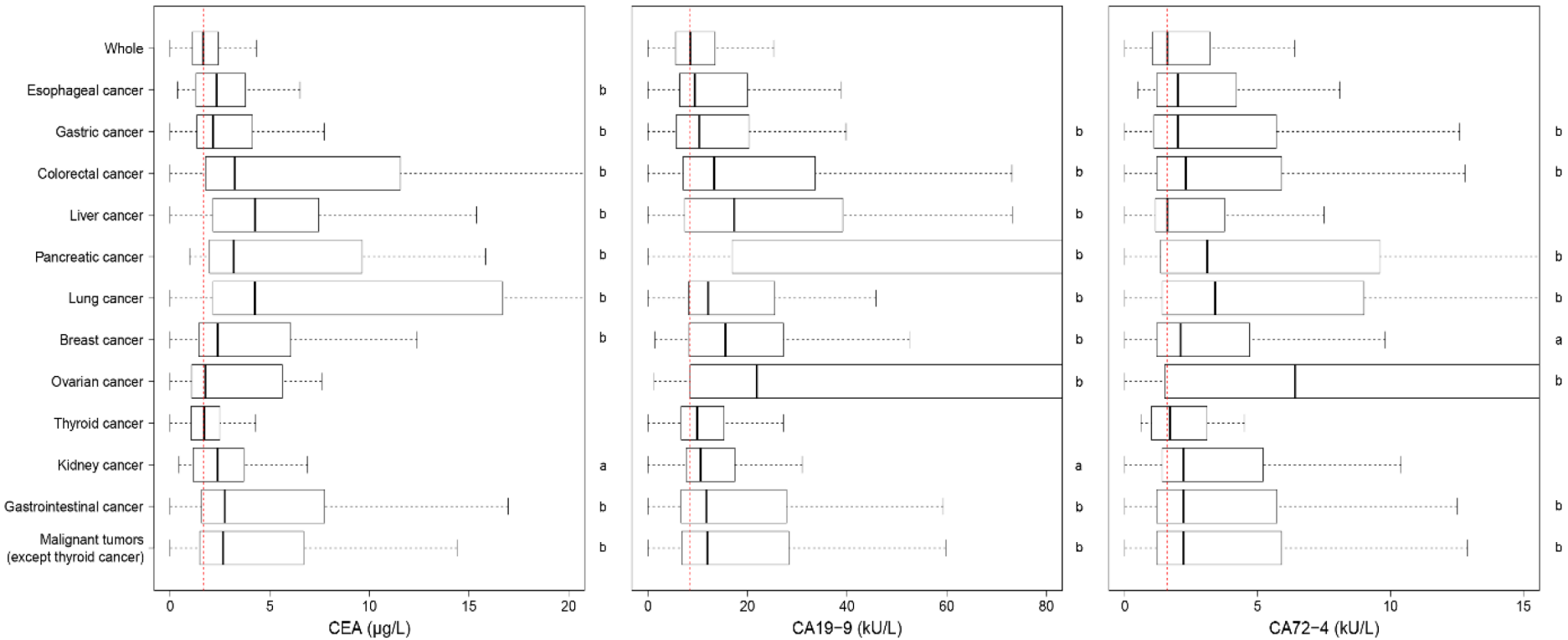

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Figure 3 Boxplots of biomarker levels of malignant tumors. The red dotted line is the biomarker level for all subjects. The *P* value was calculated between the malignant tumor patients and the subjects without any malignant tumors by the Wilcoxon test. [a]*P* < 0.05, [b]*P* < 0.01. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 72-4: Carbohydrate antigen 72-4.

biomarkers is not superior to the single biomarker, CEA.

Economic analysis of tumor biomarkers in different subgroups

The subgroup analysis under the traditional threshold is displayed in Table 12, Figures 8-10.

As we expected, for all ages, the four economic indicators of CEA in the health checkup subgroup were much higher than those in the active consultation subgroup. In the subgroup of health checkup subjects above 65 years old, all four indicators of CEA in Scheme 2 were lower than those in Scheme 1, while the total cost-effectiveness ratio in Scheme 2 was higher than that in Scheme 1 in the subgroup of health checkup subjects under 60 years. This highlights that conducting CEA testing in the health checkup for people over 65 years old is economically valuable, especially the lower cost per capita ($40.9 in Scheme 2 *vs* $146.6 in Scheme 1).

Table 11 Economic analysis of carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4 in several situations for gastrointestinal cancers

	Cut-off value	Proportion of endoscopy	Missed diagnosis rate	Total cost ($)	Cost per capita ($)	Total C/E ($)	C/E per capita ($)	Remarks
Non-blood test	-	1.000	0.000	3574.3	146.9	3574.3	146.9	Gold standard
CEA (μg/L)	0.0	1.000	0.000	3691.9	151.7	3691.9	151.7	Lowest cut-off value
	2.5	0.230	0.451	1718.1	38.7	3130.1	70.6	Highest youden index
	4.3	0.065	0.642	990.0	14.6	2767.1	40.7	Lowest total cost-effectiveness ratio
	5.0	0.049	0.674	903.1	12.1	2770.9	37.1	Traditional diagnostic cut-off value
	8.7	0.020	0.759	783.9	7.8	3246.2	32.2	Lowest total cost & lowest cost-effectiveness ratio per capita
CA19-9 (kU/L)	0.0	1.000	0.000	3755.4	154.3	3755.4	154.3	Lowest cut-off value & lowest total cost-effectiveness ratio
	20.0	0.121	0.665	1836.7	25.3	5485.7	75.5	Highest youden index
	36.9	0.033	0.787	1398.5	12.2	6578.5	57.5	Lowest total cost & lowest cost-effectiveness ratio per capita
	37.0	0.032	0.789	1405.2	12.2	6656.1	57.7	Traditional diagnostic cut-off value
CA72-4 (kU/L)	0.0	1.000	0.000	3755.4	154.3	3755.4	154.3	Lowest cut-off value & lowest total cost-effectiveness ratio
	3.4	0.231	0.623	2670.7	41.4	7083.5	109.7	Highest youden index
	6.0	0.126	0.756	2584.5	25.9	10605.1	106.2	Traditional diagnostic cut-off value
	10.5	0.064	0.833	2451.6	16.8	14709.6	100.7	Lowest total cost & lowest cost-effectiveness ratio per capita
CEA	5.0	0.069	0.601	1365.1	22.4	3419.2	56.1	Traditional diagnostic cut-off value in parallel
CA19-9	37.0							
CEA	6.9	0.036	0.676	1309.0	17.4	4034.6	53.8	Lowest cut-off value & lowest total cost-effectiveness ratio in parallel
CA19-9	69.2							
CEA	3.9	0.098	0.554	1455.6	26.7	3264.3	59.8	Lowest total cost-effectiveness ratio in parallel
CA19-9	38.1							
CEA	5.0	0.012	0.862	2433.3	13.8	17661.0	100.0	Traditional diagnostic cut-off value in serial
CA19-9	37.0							
CEA	5.4	0.042	0.689	1437.7	18.4	4621.3	59.1	Lowest cut-off value & lowest total cost-effectiveness ratio in serial
CA19-9	0.0							
CEA	2.1	0.335	0.361	2339.4	61.4	3659.5	96.1	Lowest total cost-effectiveness ratio in serial
CA19-9	0.0							
CEA	5.0	0.044	0.666	1362.9	18.7	4079.6	56.0	Traditional diagnostic cut-off value in the logistic model
CA19-9	37.0							
CEA	4.9	0.052	0.641	1341.0	19.8	3732.7	55.1	Lowest cut-off value & lowest total cost-effectiveness ratio in the logistic model
CA19-9	23.2							
CEA	2.0	0.213	0.436	1874.8	43.5	3321.5	77.0	Lowest total cost-effect-

CA19-9	33.5	iveness ratio in the logistic model

The economic indicators were total cost, cost per capita and their cost-effectiveness ratios. The cut-off value with the lowest total cost equaled that of the lowest cost-effectiveness ratio per capita. In the parallel test, any positive result of carcinoembryonic antigen (CEA) or carbohydrate antigen 19-9 (CA 19-9) was considered positive, while both of the positive results of CEA and CA19-9 were considered positive in the serial test. The logistic model was CEA × 2.02 + CA19-9 × 0.06, which made the area under the curve highest. C/E: Cost-effectiveness ratio; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4.

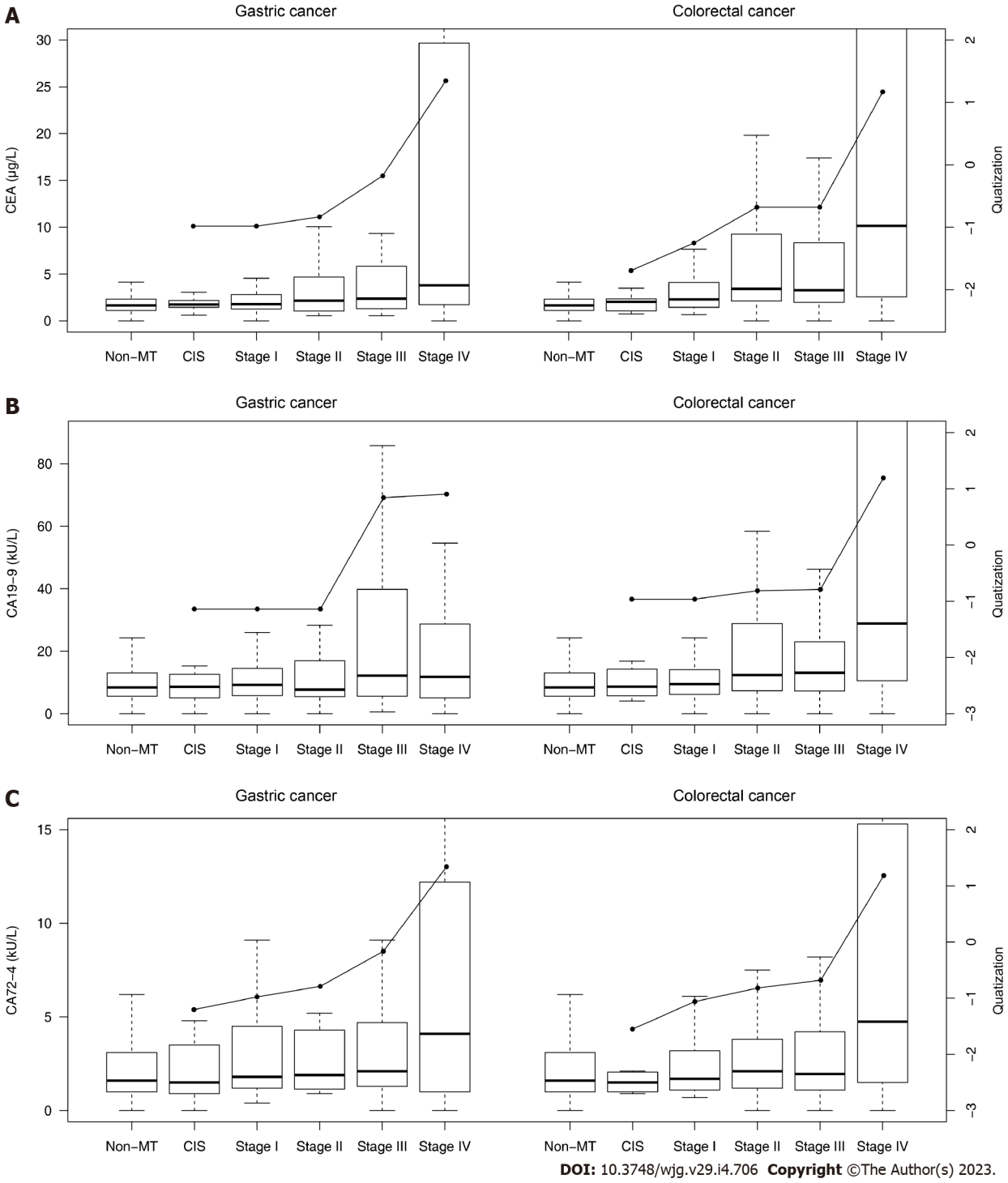

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Figure 4 Boxplots of biomarker levels for each stage of gastric cancer and colorectal cancer. A: Carcinoembryonic antigen; B: Carbohydrate antigen 19-9; C: Carbohydrate antigen 72-4. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 72-4: Carbohydrate antigen 72-4; CIS: Carcinoma *in situ*.

Table 12 Subgroup analysis of economic indicators

			Scheme 2							Scheme 1	
			Cut-off value	Proportion of endoscopy	Missed diagnosis rate	Total cost ($)	Cost per capita ($)	Total C/E ($)	C/E per capita ($)	Total cost & C/E ($)	Cost & C/E per capita ($)
CEA	Whole	Whole	5.0	0.049	0.674	903.1	12.1	2770.9	37.1	3574.3	146.9
		≥ 80 yr	5.0	0.258	0.543	381.8	45.6	835.9	99.8	584.4	152.7
		≥ 75 yr	5.0	0.240	0.635	472.1	41.9	1294.7	115.0	623.2	151.8
		≥ 70 yr	5.0	0.214	0.627	463.5	37.7	1242.1	101.2	691.5	150.9
		≥ 65 yr	5.0	0.174	0.634	486.4	31.6	1329.1	86.3	844.6	149.9
		≥ 60 yr	5.0	0.137	0.636	523.6	25.8	1438.6	71.0	1099.1	149.0
		≥ 55 yr	5.0	0.110	0.639	574.1	21.6	1588.8	59.8	1424.3	148.3
		≥ 50 yr	5.0	0.085	0.643	662.6	17.7	1854.8	49.6	1972.8	147.6
		≥ 45 yr	5.0	0.065	0.658	758.5	14.6	2220.9	42.7	2614.6	147.2
		≥ 40 yr	5.0	0.055	0.668	837.4	13.1	2520.1	39.4	3121.4	147.0
		≥ 35 yr	5.0	0.051	0.671	882.1	12.4	2684.9	37.8	3427.4	146.9
		≥ 30 yr	5.0	0.049	0.674	902.2	12.2	2764.8	37.3	3552.0	146.9
	HC	Whole	5.0	0.021	0.883	6834.3	7.7	58218.5	65.4	15277.9	146.1
		≥ 80 yr	5.0	0.238	0.000	446.3	42.5	446.3	42.5	1565.3	149.1
		≥ 75 yr	5.0	0.129	0.500	941.8	24.4	1883.7	48.7	2843.9	147.1
		≥ 70 yr	5.0	0.101	0.538	693.8	20.0	1503.3	43.3	2356.2	146.9
		≥ 65 yr	5.0	0.070	0.630	1046.6	15.1	2832.1	40.9	3755.2	146.6
		≥ 60 yr	5.0	0.049	0.744	1677.6	12.0	6542.7	46.7	5254.5	146.4
		≥ 55 yr	5.0	0.040	0.794	2611.7	10.5	12685.3	51.1	7474.5	146.3
		≥ 50 yr	5.0	0.033	0.824	3519.3	9.5	19989.5	53.8	9558.2	146.2
		≥ 45 yr	5.0	0.026	0.848	4577.8	8.5	30179.8	55.7	12010.0	146.2
		≥ 40 yr	5.0	0.023	0.871	5726.3	8.0	44326.1	61.8	13537.7	146.2
		≥ 35 yr	5.0	0.021	0.879	6443.2	7.8	53455.1	64.6	14572.3	146.1
		≥ 30 yr	5.0	0.021	0.881	6737.6	7.7	56396.5	64.7	15225.1	146.1
	AC	Whole	5.0	0.126	0.631	515.4	24.2	1397.6	65.5	1170.9	148.8
		≥ 80 yr	5.0	0.260	0.557	378.1	45.8	853.5	103.4	559.6	153.0
		≥ 75 yr	5.0	0.260	0.640	449.7	45.2	1249.2	125.5	547.0	152.7
		≥ 70 yr	5.0	0.256	0.634	439.0	44.4	1200.5	121.3	551.5	152.4
		≥ 65 yr	5.0	0.240	0.634	433.8	42.0	1186.4	114.8	574.1	152.0
		≥ 60 yr	5.0	0.219	0.624	436.5	38.9	1161.3	103.4	639.4	151.5
		≥ 55 yr	5.0	0.197	0.621	445.2	35.4	1173.3	93.4	719.0	150.9
		≥ 50 yr	5.0	0.171	0.616	471.6	31.2	1229.4	81.5	868.1	149.9
		≥ 45 yr	5.0	0.146	0.626	493.5	27.4	1319.3	73.2	1006.5	149.3
		≥ 40 yr	5.0	0.134	0.628	508.3	25.5	1367.6	68.6	1103.8	149.0
		≥ 35 yr	5.0	0.129	0.629	513.2	24.6	1383.2	66.3	1151.7	148.9
		≥ 30 yr	5.0	0.126	0.631	516.1	24.2	1400.2	65.7	1168.9	148.8
CA19-9	Whole	Whole	37.0	0.032	0.789	1405.2	12.2	6656.1	57.7	3574.3	146.9
		≥ 80 yr	37.0	0.168	0.741	494.9	33.5	1908.7	129.3	584.4	152.7

		≥ 75 yr	37.0	0.164	0.735	505.6	32.7	1906.7	123.2	623.2	151.8
		≥ 70 yr	37.0	0.137	0.761	546.5	28.5	2288.5	119.3	691.5	150.9
		≥ 65 yr	37.0	0.115	0.762	589.9	25.0	2474.1	104.7	844.6	149.9
		≥ 60 yr	37.0	0.090	0.754	633.2	21.2	2568.7	85.8	1099.1	149.0
		≥ 55 yr	37.0	0.070	0.764	736.3	18.1	3114.0	76.7	1424.3	148.3
		≥ 50 yr	37.0	0.054	0.774	913.9	15.5	4035.0	68.4	1972.8	147.6
		≥ 45 yr	37.0	0.042	0.782	1108.4	13.6	5075.1	62.4	2614.6	147.2
		≥ 40 yr	37.0	0.036	0.785	1254.6	12.7	5833.6	59.1	3121.4	147.0
		≥ 35 yr	37.0	0.033	0.789	1359.0	12.3	6434.6	58.2	3427.4	146.9
		≥ 30 yr	37.0	0.032	0.789	1398.6	12.2	6634.7	57.8	3552.0	146.9
	HC	Whole	37.0	0.014	0.917	11789.9	9.3	142719.5	112.8	15277.9	146.1
		≥ 80 yr	37.0	0.143	0.500	622.7	29.7	1245.5	59.3	1565.3	149.1
		≥ 75 yr	37.0	0.078	0.833	2187.1	18.9	13122.9	113.1	2843.9	147.1
		≥ 70 yr	37.0	0.048	0.808	1207.9	14.5	6281.3	75.3	2356.2	146.9
		≥ 65 yr	37.0	0.043	0.783	1621.1	13.8	7457.1	63.3	3755.2	146.6
		≥ 60 yr	37.0	0.032	0.808	2252.2	12.1	11711.6	62.8	5254.5	146.4
		≥ 55 yr	37.0	0.026	0.853	3855.6	11.1	26218.2	75.5	7474.5	146.3
		≥ 50 yr	37.0	0.020	0.873	5259.2	10.2	41488.9	80.5	9558.2	146.2
		≥ 45 yr	37.0	0.016	0.899	7832.3	9.6	77452.6	95.3	12010.0	146.2
		≥ 40 yr	37.0	0.014	0.909	9540.9	9.4	104950.4	103.0	13537.7	146.2
		≥ 35 yr	37.0	0.014	0.915	10937.8	9.3	128950.9	109.7	14572.3	146.1
		≥ 30 yr	37.0	0.014	0.916	11530.0	9.3	137146.2	110.7	15225.1	146.1
	AC	Whole	37.0	0.082	0.763	663.4	20.0	2793.3	84.3	1170.9	148.8
		≥ 80 yr	37.0	0.170	0.747	488.5	33.8	1929.4	133.5	559.6	153.0
		≥ 75 yr	37.0	0.180	0.731	469.9	35.2	1749.5	131.1	547.0	152.7
		≥ 70 yr	37.0	0.171	0.757	502.4	33.7	2069.9	138.9	551.5	152.4
		≥ 65 yr	37.0	0.159	0.760	503.3	32.0	2093.5	133.2	574.1	152.0
		≥ 60 yr	37.0	0.144	0.748	496.7	29.7	1967.4	117.7	639.4	151.5
		≥ 55 yr	37.0	0.126	0.753	519.6	26.9	2105.0	109.0	719.0	150.9
		≥ 50 yr	37.0	0.109	0.759	581.1	24.2	2411.0	100.4	868.1	149.9
		≥ 45 yr	37.0	0.095	0.762	620.3	21.9	2601.4	92.0	1006.5	149.3
		≥ 40 yr	37.0	0.087	0.761	644.3	20.8	2694.8	87.0	1103.8	149.0
		≥ 35 yr	37.0	0.083	0.763	659.0	20.2	2780.6	85.2	1151.7	148.9
		≥ 30 yr	37.0	0.082	0.763	663.9	20.0	2805.1	84.5	1168.9	148.8
CA72-4	Whole	Whole	6.0	0.126	0.756	2584.5	25.9	10605.1	106.2	3574.3	146.9
		≥ 80 yr	6.0	0.194	0.790	676.1	37.1	3221.6	176.7	584.4	152.7
		≥ 75 yr	6.0	0.190	0.762	627.4	36.3	2641.0	152.8	623.2	151.8
		≥ 70 yr	6.0	0.180	0.755	651.5	34.8	2661.4	142.2	691.5	150.9
		≥ 65 yr	6.0	0.173	0.749	749.3	33.4	2980.7	133.0	844.6	149.9
		≥ 60 yr	6.0	0.166	0.745	933.2	32.3	3653.5	126.5	1099.1	149.0
		≥ 55 yr	6.0	0.158	0.746	1167.6	30.9	4599.8	121.6	1424.3	148.3
		≥ 50 yr	6.0	0.147	0.749	1552.7	29.1	6194.2	116.2	1972.8	147.6
		≥ 45 yr	6.0	0.139	0.753	2003.3	27.9	8106.4	112.8	2614.6	147.2

	≥ 40 yr	6.0	0.132	0.752	2293.2	26.7	9259.2	108.0	3121.4	147.0
	≥ 35 yr	6.0	0.128	0.755	2488.4	26.2	10145.7	106.7	3427.4	146.9
	≥ 30 yr	6.0	0.127	0.755	2565.2	25.9	10489.2	106.1	3552.0	146.9
HC	Whole	6.0	0.108	0.870	18401.4	23.0	141077.7	176.0	15277.9	146.1
	≥ 80 yr	6.0	0.333	0.500	1206.6	57.5	2413.3	114.9	1565.3	149.1
	≥ 75 yr	6.0	0.190	0.667	2042.4	35.2	6127.2	105.6	2843.9	147.1
	≥ 70 yr	6.0	0.144	0.808	2375.7	28.5	12353.8	148.1	2356.2	146.9
	≥ 65 yr	6.0	0.126	0.848	4345.5	25.8	28556.3	169.7	3755.2	146.6
	≥ 60 yr	6.0	0.118	0.821	4898.1	24.5	27289.4	136.5	5254.5	146.4
	≥ 55 yr	6.0	0.119	0.843	8033.4	24.7	51212.7	157.2	7474.5	146.3
	≥ 50 yr	6.0	0.118	0.866	11985.7	24.5	89577.1	183.4	9558.2	146.2
	≥ 45 yr	6.0	0.116	0.854	13653.0	24.3	93470.7	166.2	12010.0	146.2
	≥ 40 yr	6.0	0.111	0.861	15690.0	23.5	113076.5	169.4	13537.7	146.2
	≥ 35 yr	6.0	0.109	0.866	17224.4	23.1	128608.7	172.7	14572.3	146.1
	≥ 30 yr	6.0	0.108	0.867	18051.9	23.0	135991.3	173.3	15225.1	146.1
AC	Whole	6.0	0.177	0.733	997.5	33.8	3736.5	126.8	1170.9	148.8
	≥ 80 yr	6.0	0.183	0.797	643.0	35.6	3174.8	175.8	559.6	153.0
	≥ 75 yr	6.0	0.190	0.766	558.4	36.5	2383.4	155.8	547.0	152.7
	≥ 70 yr	6.0	0.194	0.751	539.5	37.2	2165.0	149.1	551.5	152.4
	≥ 65 yr	6.0	0.202	0.739	554.2	38.2	2126.5	146.7	574.1	152.0
	≥ 60 yr	6.0	0.212	0.736	634.8	39.7	2405.9	150.4	639.4	151.5
	≥ 55 yr	6.0	0.206	0.735	694.1	38.6	2617.8	145.6	719.0	150.9
	≥ 50 yr	6.0	0.194	0.732	793.2	36.7	2963.2	137.0	868.1	149.9
	≥ 45 yr	6.0	0.186	0.736	901.9	35.4	3410.7	133.8	1006.5	149.3
	≥ 40 yr	6.0	0.182	0.731	953.6	34.6	3547.9	128.7	1103.8	149.0
	≥ 35 yr	6.0	0.179	0.732	984.8	34.1	3674.4	127.3	1151.7	148.9
	≥ 30 yr	6.0	0.177	0.733	995.6	33.9	3723.6	126.8	1168.9	148.8

The economic indicators were total cost, cost per capita and their cost-effectiveness ratios. The units of carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4 are μg/L, kU/L, and kU/L, respectively. C/E: Cost-effectiveness ratio; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4; HC: Health checkup; AC: Active consultation.

In the active consultation subgroup, the total cost-effectiveness ratio in Scheme 2 was higher than that in Scheme 1 for all ages. CA19-9 and CA72-4 had higher total cost-effectiveness ratios in almost all subgroups, different from CEA (Figures 9 and 10). This also indicates that blood tests for the active consultation group are not enough and that the necessary gastrointestinal endoscopy procedure is more important.

DISCUSSION

This study included more than 32000 subjects who received CEA, CA19-9, CA72-4, gastroscopy and colonoscopy assessments. In our study, CEA and CA19-9 again have been proved to be ideal serum biomarkers for screening GICs. The specificity of CEA and CA19-9 was approximately 95.0%-97.5% at the traditional cut-off value, which was highly consistent with previous studies[5,6]. While for the diagnostic value of CA72-4, there is a discrepancy between the results of previous literatures and our clinical practice. In our study, the specificity of CA72-4 was less than 90%, indicating that the cut-off value could be higher, which made the sensitivity even lower. If the cut-off value of CA72-4 was 10, the sensitivity and specificity of GC were 0.163 and 0.933, respectively, and the sensitivity and specificity of CRC were 0.177 and 0.935, respectively.

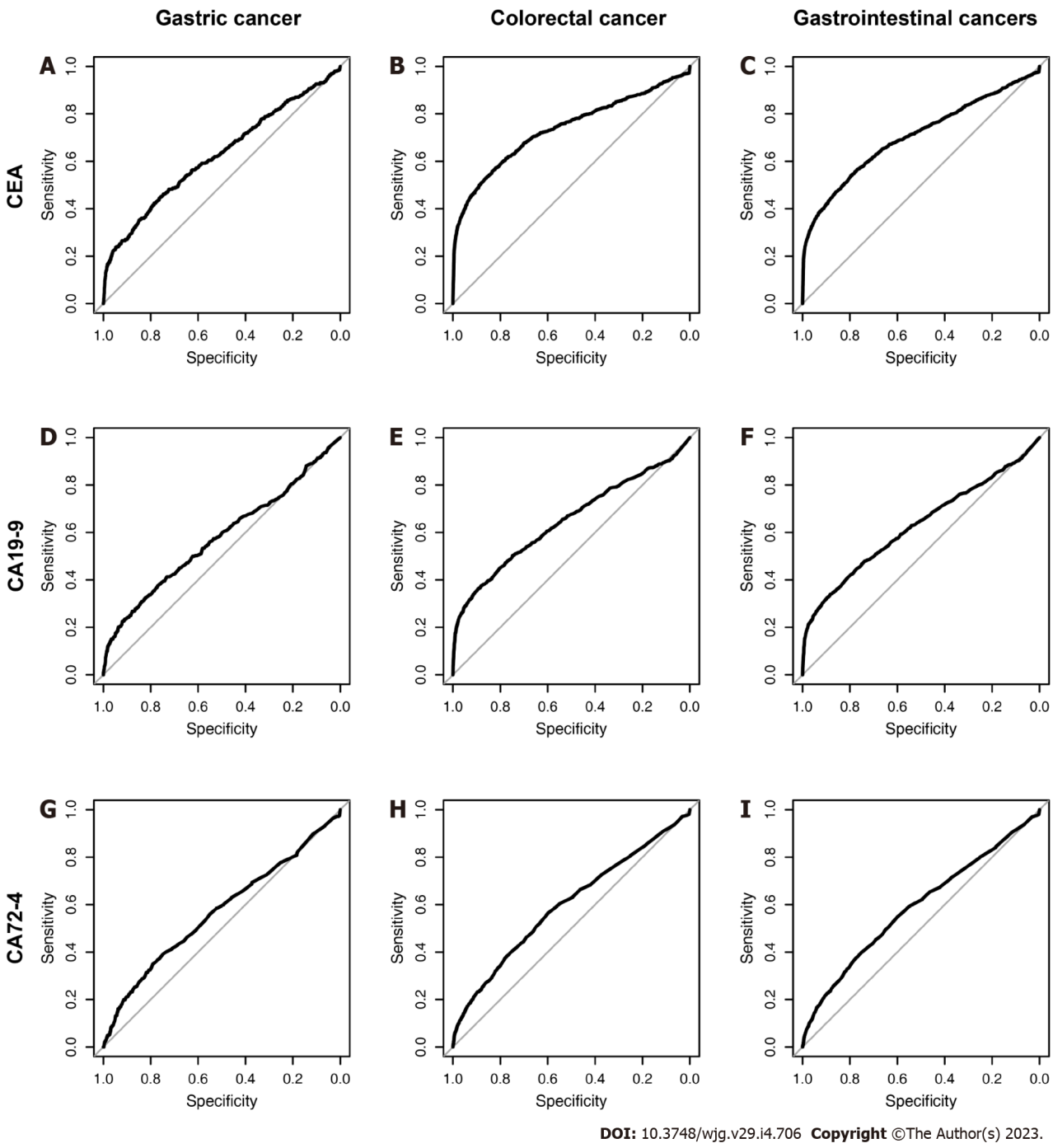

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Figure 5 Receiver operating characteristic curves of carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4 for gastric cancer, colorectal cancer and gastrointestinal cancers. A-C: Carcinoembryonic antigen; D-F: Carbohydrate antigen 19-9; G-I: Carbohydrate antigen 72-4. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 72-4: Carbohydrate antigen 72-4.

Besides the sensitivity and specificity, another important indicator is the PPV. Even for the best performing CEA, the PPV for GC was as low as 5.6% and that for CRC was only 21.2%. At the traditional cut-off value, the PPV of CA72-4 for GC was 2.3%, which meant that 97.7% of CA72-4-positive patients were false positive. The PPV also explained why there was no evidence of malignant disease in a large number of CA72-4-positive patients after a full set of auxiliary examinations. Of course, in view of the fact that the PPV is greatly affected by the prevalence, the real-world PPV would be lower. Therefore, our data on the predictive value is mainly used for comparison among the three biomarkers.

Several novel indicators are proposed to evaluate the economic value of blood markers for GICs. To calculate the economic value of a blood biomarker, it is inadequate to focus on the biomarker itself. A blood test is used as a screening test, and its significance also lies in the following gold standard test. By combining blood tests and endoscopy, the total cost and cost per capita of correctly diagnosing one case of GIC are excellent indicators, which are related to the cost, prevalence rate and sensitivity of blood

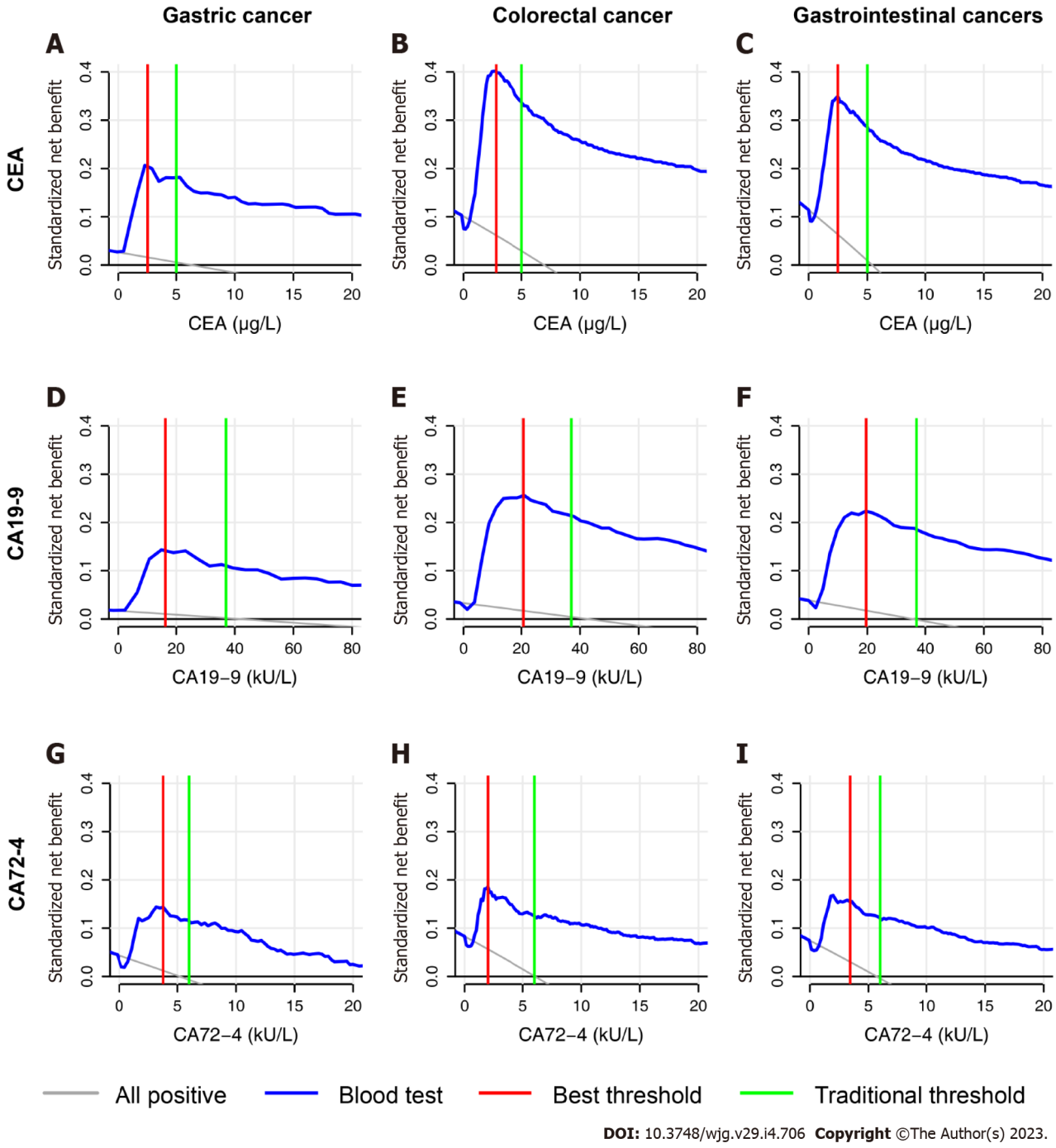

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Figure 6 Decision curves of tumor biomarkers for gastrointestinal cancers. A-C: Carcinoembryonic antigen; D-F: Carbohydrate antigen 19-9; G-I: Carbohydrate antigen 72-4. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 72-4: Carbohydrate antigen 72-4.

tests. However, these two indicators are not sufficient. If the prevalence of a disease increases, the cost per capita would also increase owing to more endoscopy examinations. It seemed that the cost increased, but the effect had actually improved even more. Therefore, it was necessary to calculate the cost-effectiveness ratio. What is the 'effect'? As a screening test, the sensitivity is its effect. The cost-effectiveness ratio is cost divided by sensitivity, which means the total cost for correctly diagnosing all subjects, including missed patients. We found that the total cost and the cost-effectiveness ratio per capita are positively correlated and change synchronously. Through our economic research, we have discovered the impacts of the order of gastrointestinal endoscopy and diagnostic thresholds on economic benefits. It is also clear that the economic value of combined blood biomarkers is not as good as that of the single CEA. Subgroup analysis shows that CEA had qualified diagnostic value for health checkup subjects above 65 years old.

In this study, only the subjects who received CEA, CA19-9, CA72-4, gastroscopy and colonoscopy were included. These inclusion criteria avoided or reduced several biases, such as workup bias, spectrum bias and measurement bias. For example, all of the included cases were examined by the gold

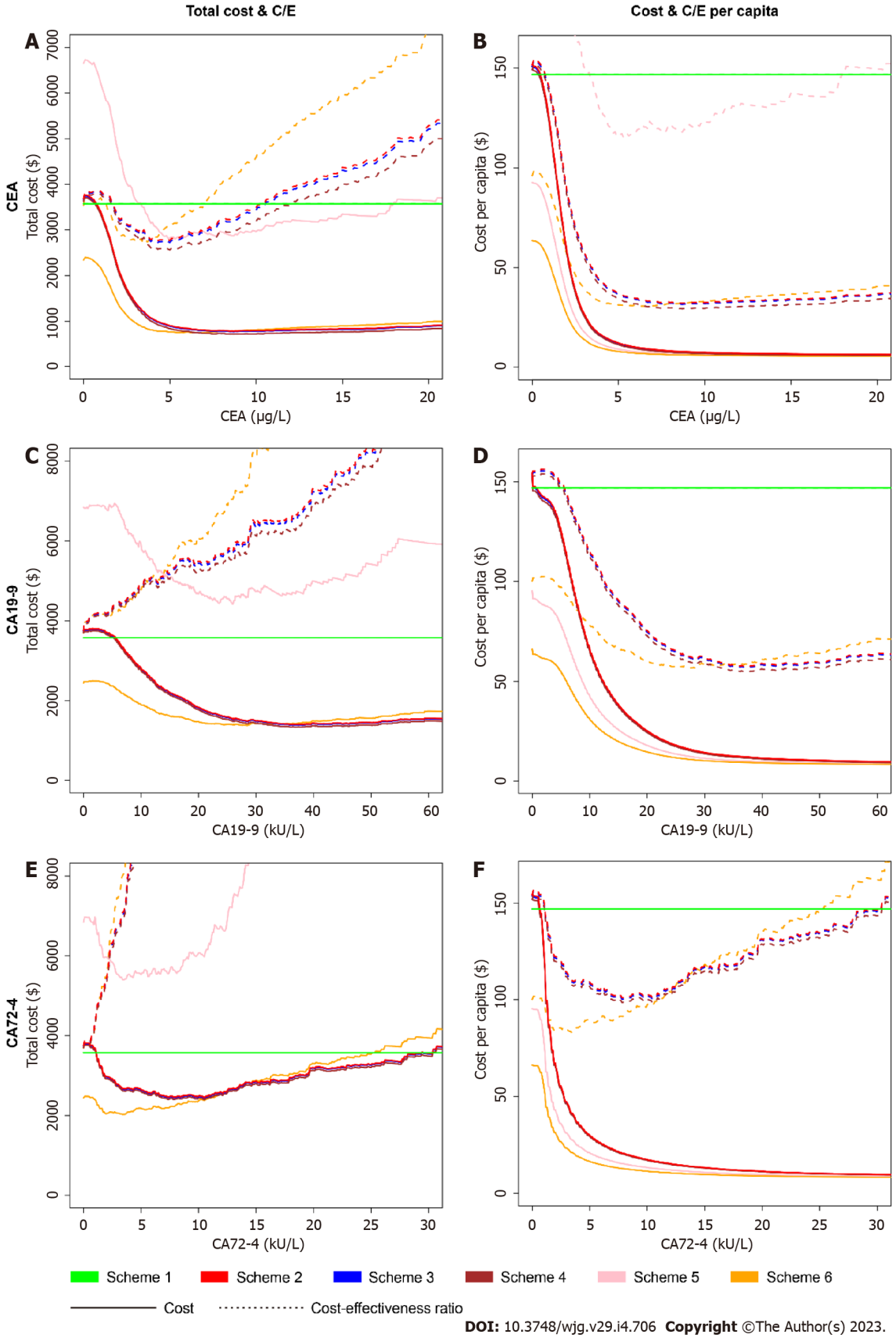

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Figure 7 Economic analysis of tumor biomarkers in six schemes for gastrointestinal cancers. A and B: Carcinoembryonic antigen; C and D: Carbohydrate antigen 19-9; E and F: Carbohydrate antigen 72-4. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 72-4: Carbohydrate antigen 72-4.

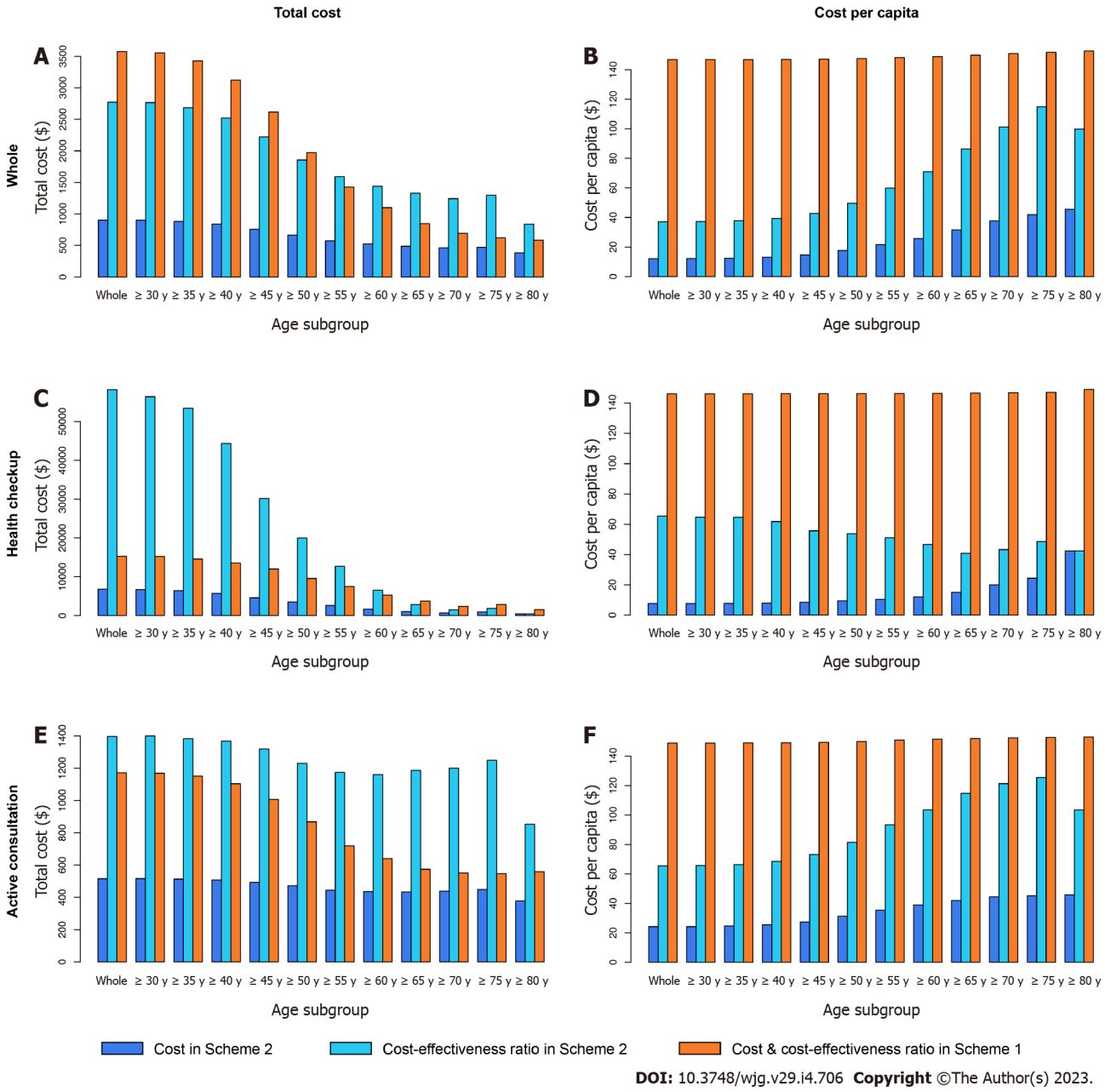

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Figure 8 Bar plots of subgroup analysis of economic indicators for carcinoembryonic antigen. A and B: Whole; C and D: Health checkup; E and F: Active consultation.

standard test, so there was no situation in which the subject with negative blood test results was not examined with the gold standard test. But on the other hand, the inclusion criteria led to an inevitable selection bias because the subjects undergoing gastrointestinal endoscopy are those with a high risk of digestive diseases, and the incidences of GC and CRC in this study were higher than that in the real world[15]. Many people undergo only blood tests but not gastrointestinal endoscopy when receiving a health checkup. As a result, some early GIC patients with normal CEA, CA19-9, CA72-4 Levels were not included. If these patients were included, the number of false negative subjects might have increased, and the sensitivity would have further decreased.

The advantages of this study are its continuous inclusion of subjects, use of the cohort study inclusion method (not case-control study), large sample size, inclusion of multiple tumors and use of multiple indicators. Especially for CA72-4 test, our sample size exceeded the sum of all previous reported studies. The comparison among multiple indicators highlighted the shortcomings of the diagnostic and economic value of CA72-4. In particular, the results of the classic markers CEA and CA19-9 were consistent with previous studies. We also proposed a new evaluation method for the economic efficiencies of tumor biomarkers for GIC and provided a reference for medical insurance policies.

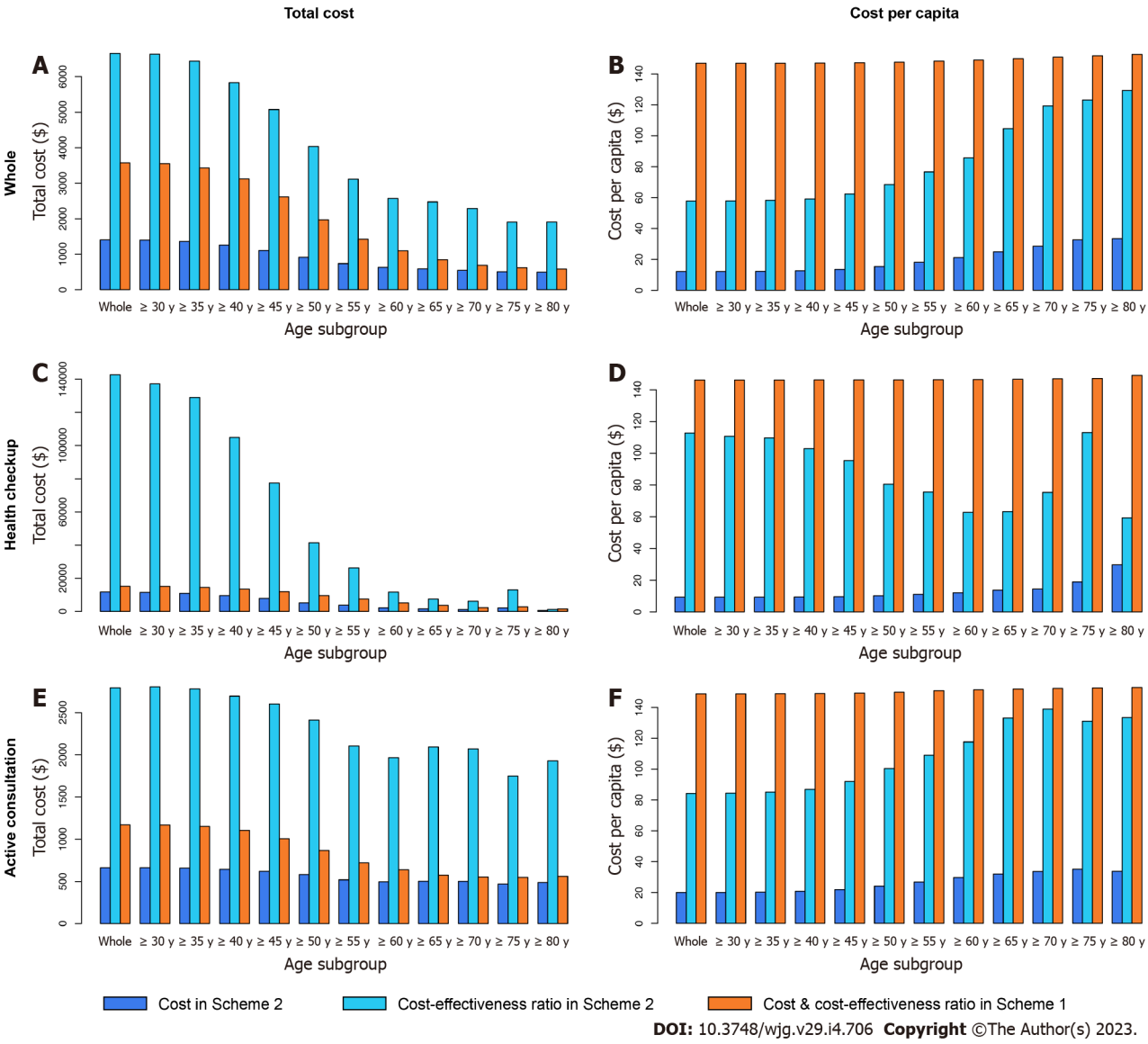

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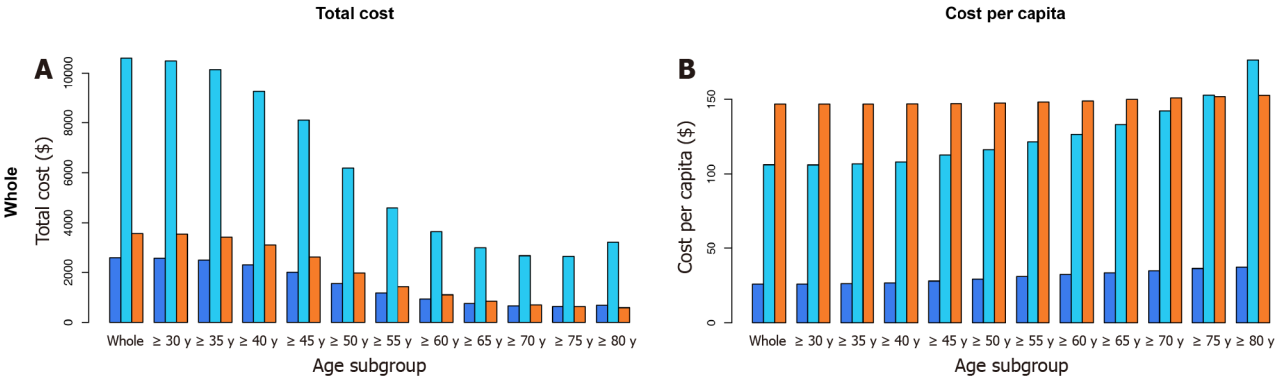
Figure 9 Bar plots of subgroup analysis of economic indicators for carbohydrate antigen 19-9. A and B: Whole; C and D: Health checkup; E and F: Active consultation.

CONCLUSION

CEA had qualified diagnostic value for CRC and superior economic value for GICs, especially for health checkup subjects above 65 years old. CA72-4 was not suitable as a diagnostic biomarker.



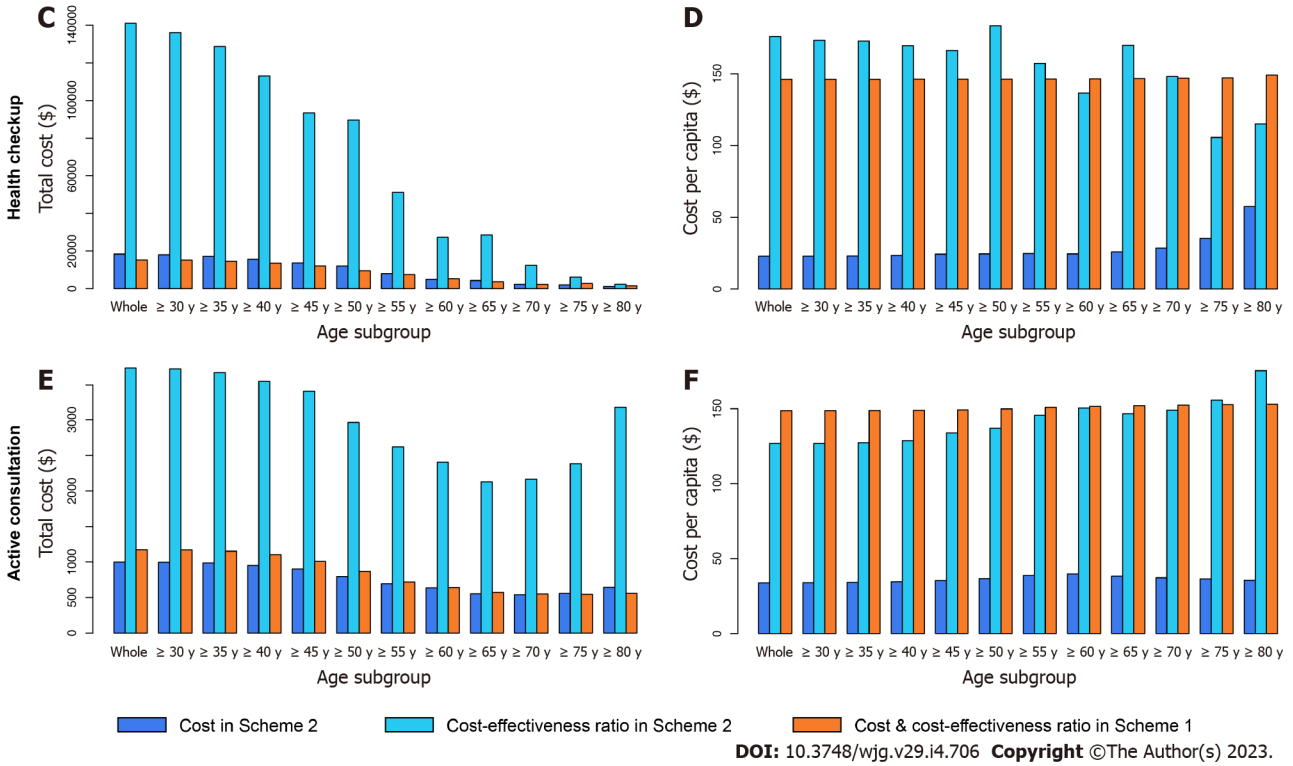


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Figure 10 Bar plots of subgroup analysis of economic indicators for carbohydrate antigen 72-4. A and B: Whole; C and D: Health checkup; E and F: Active consultation.

ARTICLE HIGHLIGHTS

Research background

Studies showed that blood carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) could be used to diagnose gastric cancer (GC) and colorectal cancer (CRC). Blood CA72-4 could be a potential biomarker to diagnose GC and CRC. A positive result in blood test would lead the subject to undergo further examinations.

Research motivation

Large-scale clinical application showed an extremely high false positive rate of CA72-4 for diagnosis, which leads to the waste of medical resources and heave social medical burden. The massive data and real-world diagnostic cohorts make it possible to further explore the diagnostic and economic value of biomarkers.

Research objectives

Through a real-world diagnostic cohort, we aimed to reassess the diagnostic and economic value of CEA, CA19-9, and CA72-4 for gastrointestinal malignant tumors in a large sample.

Research methods

Data from patients the medical examination center, outpatient department or inpatient department of Zhongshan Hospital of Fudan University from October 2006 to May 2018 were retrospectively evaluated. Four economic indicators were used to evaluate the economic value of tumor biomarkers. The diagnostic value of the three biomarkers was further evaluated.

Research results

The clinical benefits of CEA were higher than those of CA19-9, while the clinical benefits of CA72-4 were the lowest. The combination of biomarkers in the CRC and gastrointestinal malignant tumors significantly increased the AUC by less than 0.3, while that in GC did not. Compared to the economic indicators of the single biomarker CEA, the combination of biomarkers is not superior. At the threshold of 1.8 μg/L to 10.4 μg/L, all four indicators of CEA were lower than those in the scheme that conducted gastrointestinal endoscopy only. Subgroup analysis implied that the health checkup of CEA for people above 65 years old was economically valuable.

Research conclusions

CEA had qualified diagnostic value for CRC and superior economic value for gastrointestinal cancers, especially for health checkup subjects above 65 years old while CA72-4 was not suitable as a diagnostic biomarker.

Research perspectives

In real world, many people undergo only blood tests but not gastrointestinal endoscopy when receiving a health checkup. Those undergone gastrointestinal endoscopy were at a higher risk of digestive diseases, which leads to an inevitable selection bias. Future researches may emphasize on the involvement of patients with normal CEA, CA19-9, CA72-4 Levels to decrease the number of false negative subjects.

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FOOTNOTES

Author contributions: Liu HN, Yao C, and Wang XF contributed equally to this work; Liu HN, Wu H, and Liu TT conceived and designed the experiments; Liu HN, Yao C, and Wang XF drafted the manuscript; Liu HN, Yao C, Wang XF, and Pan D extracted the data; Liu HN, Zhang NP, and Chen YJ performed the statistical analyses; Zhao GP and Shen XZ revised the article; all authors finished reading and approving the final manuscript of this study.

Institutional review board statement: The study was reviewed and approved by the Zhongshan Hospital of Fudan University Institutional Review Board (Approval No. B2018-234).

Informed consent statement: The informed consent was waived from the patients.

Conflict-of-interest statement: All the authors have no conflict of interest related to the manuscript.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at liu.taotao@zs-hospital.sh.cn. Participants gave informed consent for data sharing.

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ORIGINAL ARTICLE

Retrospective Study

Feasibility and efficacy of endoscopic purse-string suture-assisted closure for mucosal defects induced by endoscopic manipulations

Ming-Ming Li, Yi Zhang, Fang Sun, Man-Xiu Huai, Fei-Yu Zhang, Chun-Ying Qu, Feng Shen, Zheng-Hong Li, Lei-Ming Xu

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Abstract

BACKGROUND

Large or transmural defects induced by gastrointestinal endoscopic manipulations are difficult to close, although complete closure is recommended for better recovery. Endoscopic purse-string assisted suturing (EPSS) has been used in clinical practice and has proven to be an effective and safe technique for the closure of large mucosal defects. However, details regarding the efficacy of endoscopic pre-purse-string suture (P-EPSS) are unknown, especially that it offers several advantages over conventional EPSS (C-EPSS).

AIM

To elucidate the outcomes of EPSS-assisted closure in different clinical situations, and evaluate the efficacy of P-EPSS.

METHODS

This retrospective observational study included a total of 180 patients who underwent closure assisted by P-EPSS (n = 63) or C-EPSS (n = 117) between July 2014 and June 2020. The P-EPSS and C-EPSS groups were compared and the intergroup differences in aspects such as the lesion size, location, and morphology, incidence of complete closure, intraoperative perforation, and delayed adverse events were evaluated. Data on the features and clinical course of cases with adverse events were collected for further analysis.

RESULTS

Patients with lesion size larger than 3 cm, lesions located at the fundus of stomach, or submucosal tumors originating from the deep mucosa were more likely to undergo P-EPSS-assisted closure. The P-EPSS group showed a significantly higher proportion of intraoperative perforation (56% *vs* 17%) and a much

shorter procedure time (9.06 ± 6.14 min *vs* 14.84 ± 7.25 min). Among adverse events, the incidence of delayed perforation (5% *vs* 4%; P = 0.82) and delayed bleeding (3% *vs* 4%; P = 0.96) did not differ significantly between the groups. Multivariate analysis revealed that lesions with incomplete closure [odds ratio (OR) = 21.33; 95% confidence interval (CI): 5.45-83.45; P < 0.01] or size greater than 3 cm (OR = 3.14; 95%CI: 1.08-9.18; P = 0.039) showed a statistical tendency to result in an increase in delayed adverse events.

CONCLUSION

The present study revealed that EPSS could achieve secure complete closure of mucosal defect. P-EPSS could shorten the procedure and yield complete closure of mucosal defects. Rather than closure-type selection, incomplete closure or lesion size larger than 3 cm were associated with worse outcomes.

Key Words: Endoscopic purse-string suture; Mucosal defect; Endoscopic full-thickness resection; Endoscopic submucosal dissection

Core Tip: Endoscopic purse-string assisted suturing (EPSS) has proven to be an effective and safe technique for the closure of large mucosal defects. Endoscopic pre-purse-string suture (P-EPSS) is recently introduced and offers several advantages over conventional endoscopic purse-string suture (C-EPSS). We found that the novel method could offer several advantages over C-EPSS. This retrospective observational study included a total of 180 patients who underwent P-EPSS (n = 63) or C-EPSS (n = 117), and evaluate the feasibility and efficacy of P-EPSS-assisted closure in different clinical situations. In conclusion, EPSS could achieve secure complete closure of mucosal defect. P-EPSS could shorten the procedure and yield complete closure of mucosal defects rather than the C-EPSS closure-type.

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INTRODUCTION

The endoscopic purse-string assisted suturing (EPSS) method has been used in clinical practice for nearly 20 years and has proven to be an effective and safe technique for the closure of large mucosal defects induced by endoscopic manipulations[1-5]. Inspired by the surgical purse-string suture strategy, an endoloop and repositionable clips have been successfully combined to manage intraluminal wounds *via* an endoscope[6-10]. However, some defects are difficult to resect and are expected to induce perforation during endoscopic manipulations, necessitating the application of an endoscopic pre-purse-string suture (P-EPSS) procedure. This novel suture method was first described by Wu *et al*[11] and was usually applied in cases involving exposed endoscopic full-thickness resections (EFTRs). At our medical center, we expanded the indications for P-EPSS and attempted to use it in more procedures such as endoscopic submucosal dissection (ESD) and endoscopic submucosal excavation (ESE) of big defects, potentially simplifying the process of closure and avoiding the possibility of postoperative adverse events.

To our knowledge, no previous reports have described the advantages of P-EPSS over conventional EPSS (C-EPSS). Furthermore, previous clinical studies on the application of EPSS usually had small sample sizes and mainly focused on providing detailed descriptions of endoscopic procedures rather than assessing the feasibility and efficacy of the technique[12-15]. Therefore, we conducted a large-scale analysis of case series focusing on the effectiveness and safety of EPSS. We compared the differences between the P-EPSS and C-EPSS group in many aspects and tried to illustrate the considerations involved in choosing the appropriate closure type according to the defect's clinical characteristics.

MATERIALS AND METHODS

Study design

This was a retrospective observational study performed in accordance with the Helsinki Declaration. It was approved by the Ethics Committee at Xinhua Hospital (approval number: XHEC-C-2018-109), Shanghai Jiaotong University School of Medicine. Written informed consent for the procedures was obtained from all patients.

Eligible patients

A total of 180 consecutive patients with large mucosal defects who underwent C-EPSS or P-EPSS at our institute from July 2014 to June 2020 were included in the study. We compared the findings between the P-EPSS group (n = 63) and C-EPSS group (n = 117) for further analysis. All patients had undergone different endoscopic manipulations (ESD, ESE, or exposed EFTR), and the endoscopic suture method was applied to close large mucosal defects with or without transmural defects. All the procedures were completed by four chief physicians who had more than 10 years of experience in performing endoscopic operations. The patient database included data pertaining to patient demographics, location and size of the mucosal defects after resection, procedure time, successful closure rate, delayed adverse events, duration of hospitalization, and total cost of hospitalization.

C-EPSS and P-EPSS procedures

The endoscopic manipulation always started with the mucosa marking procedure, in which markings were placed 3 to 5 mm outside the circumference of the lesion. After injection of epinephrine diluted in saline solution (1:100000) into the submucosal layer to raise the submucosa, endoscopists performed the resection procedure. The C-EPSS procedure is a common method used for closure of defects. After dissection of precancerous lesions or tumor specimens, an endoloop and clips were inserted simultaneously into the location of defect through the therapeutic endoscope for complete closure. Single-channel or double-channel endoscopy was used according to the specific defect status. The endoloop was anchored onto the full thickness of the defect's distal margin with the clip, followed by insertion of three to six additional clips to anchor the endoloop at different sides of the margin. Finally, the endoloop was tightened by slightly pulling all the edges together. Other clips were used if any clip was not accurately positioned or the purse-string suture was not tight.

The P-EPSS procedure was always used when defects were difficult to resect and expected to induce perforation. In these cases, to reduce difficulties in manipulation in the suturing process, decrease the entry of gas into the abdominal cavity, and avoid postoperative adverse events, the endoloop and clips were advanced around the defect before the end of the dissection phase; this procedure was routinely performed with one or two clips. The remaining clips were anchored around the defects when the lesions were resected. Tightening of the endoloop was immediately performed after placement of the clips. This procedure was named P-EPSS. An example of the closure procedure is shown in Figures 1A-I and the Video(Supplementary material).

The procedure time of the P-EPSS-assisted closure was defined as the time from the usage of the first clip in the operation to completion of the closure, but the time required for the resection procedure was excluded (as shown in Figures 1C and 1F-I). Exclusion of the time required for the resection procedure was considered appropriate due to the differences in the difficulty levels in the operation.

Postoperative management

After the manipulations, the patients received drug therapy and were fasted for 2-4 d; body temperature was monitored daily, and postoperative adverse events were recorded as required. After evaluating the results of blood examinations and abdominal radiographs, the patients were allowed to drink, which was followed by a liquid diet. Proton pump inhibitors (rabeprazole 20 mg/d, lansoprazole 30 mg/d, or esomeprazole 20 mg/d) were administered for 1 mo if the patients had undergone upper gastrointestinal (GI) tract surgeries. Prophylactic antibiotics were routinely used in patients who showed intraoperative perforation and in patients with elevated white blood cell counts. If the patients showed good outcomes, they were discharged after 3-5 d. The first clinical follow-up was performed 3-6 mo after the manipulation, and then annually after the first year. The follow-up assessments included evaluation of digestive symptoms and gastro/enteroscopy.

Measured outcomes

The success rate of complete closure, incidence of adverse events (delayed perforation and bleeding), procedure time, duration of hospitalization, and total costs were analysed as outcomes of EPSS-assisted closure of mucosal defects and compared them between the P-EPSS and C-EPSS groups. The definition of perforation required elucidation in advance and included intraoperative and delayed perforation. The former was defined as a perforation observed before the end of the endoscopic manipulation, indicating the formation of a transmural defect during the operation; the latter was defined as any perforation found thereafter. Delayed bleeding was defined as hematemesis or melena requiring endoscopic hemostasis after the procedure. We did not treat adverse events occurring during the

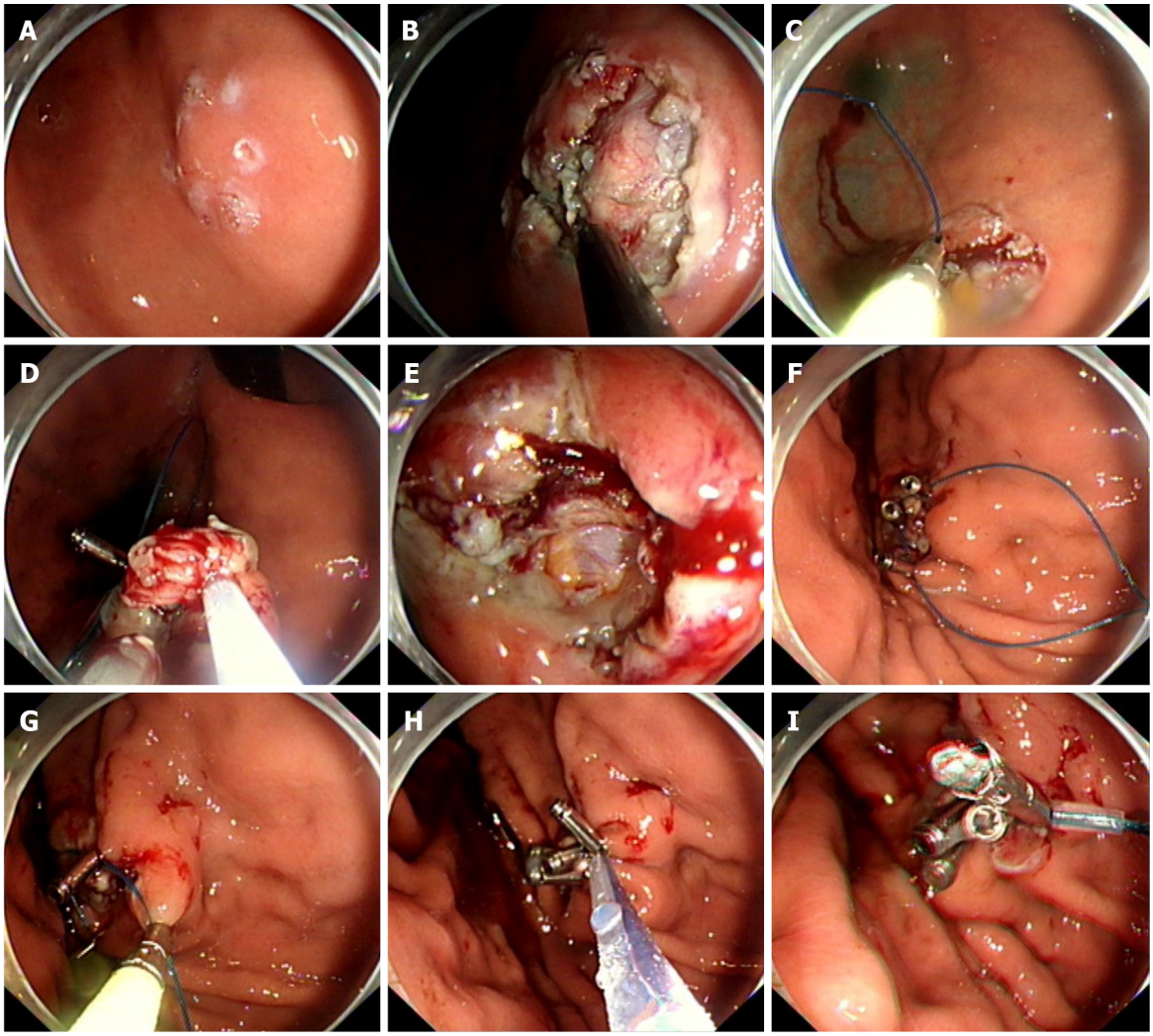

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Figure 1 Schematic figure of the pre-endoscopic purse-string assisted suturing procedure. A: Mucosa marking step: Dots were marked around the tumor; B: Expose the lesion; C: Pre-endoscopic purse-string assisted suturing by using an endoloop and a metal clip; D and E: Intraoperative perforation in exposed endoscopic full-thickness resection; F-I: Immediate closure of the perforation by tightening endoloop.

endoscopic procedure, such as intraoperative perforation in exposed EFTR, as events; only delayed adverse events such as perforations or bleeding appearing after the end of closure were treated as events. We collected data on the features and clinical course of cases with adverse events, and analyzed associations between the unsatisfactory outcomes and defect features (complete closure or not, location, size, morphology, and closure type).

Statistical analyses

Categorical variables were analyzed using Pearson's chi-squared test or Fisher's exact test, as appropriate. For instance, as listed in Table 1, we used Pearson's chi-squared test to analyze the predictors when using the P-EPSS method. Continuous variables were analyzed using the student *t* test. The data for duration and cost of hospitalization were analyzed and summarized in Table 2. *P* values < 0.05 were considered statistically significant. All data were analyzed using the SPSS for Windows statistical software package (version 22.0; SPSS, Chicago, IL, United States).

RESULTS

Baseline characteristics

Patient details are described in Table 3. During the study period, 117 patients underwent C-EPSS, while 63 patients underwent P-EPSS for closure of defects induced by endoscopic manipulations. The ratio of different endoscopic manipulations (exposed EFTR:ESE:ESD) used in the study was approximately 1:1:3 (36:32:112), but this ratio showed no significant difference between the P-EPSS and C-EPSS groups (*P* >

Table 1 Predictors for closure of mucosal defects using endoscopic pre-purse-string suture method

Predictors	P-EPSS/C-EPSS (*n*)	Odds ratio	95%CI	*P* value
Location (%)				
Fundus	35/44	2.07	1.11-3.86	0.03[1]
Others	28/73	1		
Defect size				
≥ 3 cm	44/8	31.55	12.86-77.40	< 0.01[1]
< 3 cm	19/109	1		
Morphology				
Deep SMT	36/40	2.57	1.37-4.81	< 0.01[1]
Others	27/77	1		

[1]Statistically significant.
P-EPSS: Endoscopic pre-purse-string suture; C-EPSS: Conventional endoscopic purse-string suture; SMT: Submucosal tumor; CI: Confidence interval.

Table 2 Outcomes of purse-string suture according to different closure type

Outcomes	Total	P-EPSS	C-EPSS	*P* value
Complete closure, *n* (%)	169 (94)	59 (94)	110 (94)	0.80
Intraoperative perforation, *n* (%)	55 (31)	35 (56)	20 (17)	< 0.01[1]
Delayed adverse events, *n* (%)				
Delayed perforation	8 (4)	3 (5)	5 (4)	0.82
Delayed bleeding	7 (4)	2 (3)	5 (4)	0.96
Operation time (min), mean ± SD	12.82 ± 7.83	9.06 ± 6.14	14.84 ± 7.25	< 0.01[1]
Fasting period (d), median (range)	2 (1-5)	2 (1-5)	2 (1-5)	0.88
Hospital stay (d), median (range)	6 (4-11)	6 (4-10)	6 (4-11)	0.87
Total cost (dollars), mean ± SD	2481 ± 445	2448 ± 365	2498 ± 418	0.47

[1]Statistically significant.
SD: Standard deviation; P-EPSS: Endoscopic pre-purse-string suture; C-EPSS: Conventional endoscopic purse-string suture.

0.05). In terms of the location, more than three-quarters of the defects were located in the stomach, especially in the part of the fundus (44%). The mean defect size was 2.5 ± 1.46 cm. The difference in defect size between the P-EPSS and C-EPSS groups was significant (3.6 ± 1.13 cm *vs* 2.5 ± 0.68 cm, $P < 0.05$). In terms of defect morphology, more than 70% of the defects were submucosal tumors; 42% were submucosal tumors (SMTs) located in the deep muscularis propria and 31% were superficial SMTs. The size and morphology of the defects in the two groups were different, and a detailed analysis of these characteristics is provided in the next section.

P-EPSS predictors related to the defect's clinical features

As mentioned above, many factors, including the defect's position, size, and morphology, could indicate and predict the choice of the P-EPSS method. In terms of position, more than half (54%) of the defects in the P-EPSS group were located at the fundus of the stomach, which was much higher than the corresponding proportion in the C-EPSS group (38%). In terms of the size, the proportion of cases with defects larger than 3 cm in the P-EPSS group was significantly more than the proportion of cases with defects < 3 cm in size ($P < 0.01$), indicating a significant trend toward the use of P-EPSS to close large defects. We designed a histogram to illustrate the choice of closure type according to the defect size, as shown in Figure 2. In terms of the morphology, no significant increase was observed in the proportion of cases involving P-EPSS in relation to the morphology of SMTs (33% *vs* 41%, $P > 0.05$). The difference between the P-EPSS and C-EPSS groups was significant only for patients with SMTs originating from the muscularis propria (57% *vs* 34%, $P < 0.01$). In terms of differences in endoscopic manipulations, no significant increase was observed in the proportion of P-EPSS cases in the exposed EFTR group (42% *vs*

Table 3 Baseline characteristics of the cases in this study

Patients detail	Total (*n* = 180)	P-EPSS (*n* = 63)	C-EPSS (*n* = 117)	*P* value
Age (yr), mean (range)	60 (38-78)	62 (42-76)	60 (38-78)	0.19
Sex, *n* (%)				0.75
Female	112 (62)	38 (60)	74 (63)	
Male	68 (38)	25 (40)	43 (37)	
Manipulation, *n* (%)				0.40
EFTR	36 (20)	15 (24)	21 (18)	
ESE	32 (18)	13 (21)	19 (16)	
ESD	112 (62)	35 (55)	77 (66)	
Location, *n*(%)				0.10
Stomach				
Fundus	79 (44)	35 (56)	44 (38)	
Body	45 (25)	11 (17)	34 (29)	
Antrum	22 (12)	8 (13)	14 (12)	
Colon and rectum	34 (19)	9 (14)	25 (21)	
Defect size (cm), mean ± SD	2.89 ± 1.46	3.6 ± 1.13	2.5 ± 0.68	0.02[1]
Morphology, *n*(%)				0.01[1]
Deep SMT	76 (42)	36 (57)	40 (34)	
Superficial SMT	55 (31)	15 (24)	40 (34)	
Precancerous lesions	49 (27)	12 (19)	37 (32)	

[1]Statistically significant.
SD: Standard deviation; SMT: Submucosal tumor; ESD: Endoscopic submucosal dissection; ESE: Endoscopic submucosal excavation; EFTR: Endoscopic full-thickness resection; P-EPSS: Endoscopic pre-purse-string suture; C-EPSS: Conventional endoscopic purse-string suture.

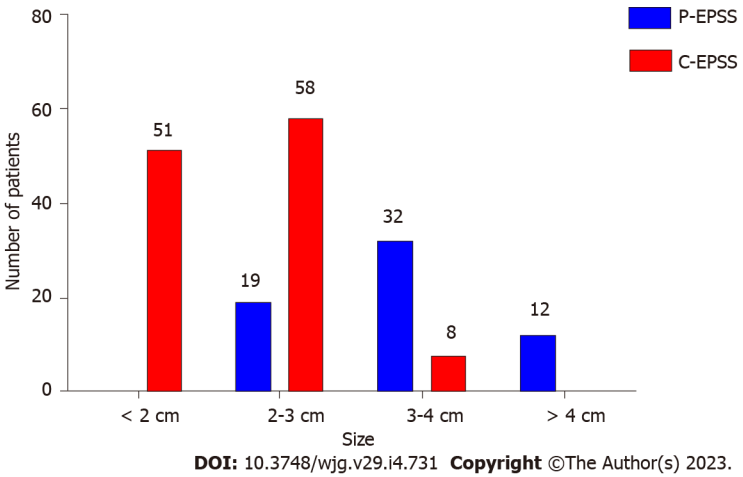

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Figure 2 Histogram of the choice of closure type according to the defect size. P-EPSS: Endoscopic pre-purse-string suture; C-EPSS: Conventional endoscopic purse-string suture.

33%, $P > 0.05$).

Next, we performed multivariate analysis to identify predictors for P-EPSS. Defect location at the fundus of the stomach [odds ratio (OR) = 2.07; 95% confidence interval (CI): 1.11-3.86; $P = 0.03$], defect size larger than 3 cm (OR = 31.55; 95%CI: 12.86-77.40; $P < 0.01$), and SMTs originating from the deep mucosa (OR = 2.57; 95%CI: 1.37-4.81; $P < 0.01$) were significantly associated with a high rate of P-EPSS (Table 1).

Comparison of treatment outcomes between the P-EPSS and C-EPSS groups

Table 2 presents a comparison of treatment outcomes between the P-EPSS and C-EPSS groups. The proportion of complete closure assisted by purse-string sutures was 94% and was similar between the two groups. Patients with incomplete closure received conservative treatment and eventually recovered from the trauma. None of them required alternative surgical repair procedures. While more patients from the P-EPSS group were inclined to experience intraoperative perforation (56% *vs* 17%, $P < 0.01$), the procedure time was significantly shorter in the P-EPSS group (9.06 ± 6.14 min *vs* 14.84 ± 7.25 min, $P < 0.01$).

In the assessment of adverse events, the two groups showed no significant differences in the proportions of delayed perforation (5% *vs* 4%; $P = 0.82$) and delayed bleeding (3% *vs* 4%; $P = 0.96$). Cases of adverse events were successfully managed by endoscopic treatment and conservative therapy. After the treatment, the fasting period, duration of hospitalization, and total cost of hospitalization were similar between the two groups.

Analysis of risk factors for delayed adverse events

The features and clinical course of the 15 cases showing delayed adverse events are shown in Table 4, and the results of the logistic regression analysis are shown in Table 5. Multivariate analysis revealed that incomplete closure of the defect was the main independent predictor for an increased number of delayed perforation or bleeding events, and it showed approximately 95% significant increase in delayed adverse events (OR = 21.33; 95%CI: 5.45-83.45; $P < 0.01$). Moreover, cases with defect size more than 3 cm showed a statistical tendency toward an increase in delayed adverse events (OR = 3.14; 95%CI: 1.08-9.18; $P = 0.039$). However, defect position (fundus or others) or morphology (SMT or precancerous lesions) as well as the closure type selected (P-EPSS or C-EPSS) were not related to significant differences in delayed adverse events.

DISCUSSION

With the development and popularization of endoscopic techniques, more GI diseases can be detected early and manipulated using minimally invasive methods[16-18]. GI endoscopy procedures such as ESD, ESE, EFTR, and submucosal tunnel endoscopic resection are widely used for the treatment of precancerous lesions or SMTs within the GI tract[19-23]. However, their increasing usage has led to the problem of achieving successful complete closure of the mucosal defects induced by different endoscopic manipulations.

Clips can be applied to effectively manage small defects, but they are too small to work well for large mucosal defects. More importantly, tissue approximation with full-thickness closure is technically impossible with clips, and clips may prematurely drop off the mucosa due to peristalsis and the radial forces of large post-EFTR defects, resulting in delayed perforation and severe complications. Furthermore, clip-closure methods appear to be strongly operator-dependent[9,24,25].

These factors have necessitated the development of new techniques. As reported in one study, various techniques have been used for closure of large defects (especially for exposed defects after EFTR), and these mainly consist of clip- and endoloop-assisted closure methods[26]. For instance, multiple studies reported that over-the-scope-clip (OTSC) application, which can close defects with serosa-to-serosa apposition, unlike mucosa-to-mucosa apposition, can successfully close defects with long-term reliability[27-30]. However, OTSCs can close GI defects only up to 2.5-3 cm in size[31,32], and the OTSC system shows limited effectiveness in some anatomic sites, such as the pylorus and the proximal esophagus. In addition, the edema and tissue folding associated with the usage of OTSCs can potentially narrow the lumen[33], and the OTSC technique is expensive, which can increase the financial burden on patients.

Purse-string assisted suturing has been widely used and is a financially feasible approach in cases with large defects induced by endoscopic manipulations. However, previous studies on the efficacy of EPSS methods evaluated a limited source of cases. Moreover, most of these studies focused on providing a detailed introduction of the procedure for purse-string sutures rather than performing a detailed analysis of its feasibility and efficacy. Wang *et al*[12] reported that the purse-string suture for colonic mucosal defects could be successfully completed using a single-channel endoscope. They described the detailed procedure of the EPSS method and concluded that no severe complications were recorded in all 18 cases. Kato *et al*[34] summarized their findings for duodenal ESD and found that complete closure of the mucosal defect by the EPSS method was relatively easy and involved a reduced delayed adverse event rate. Other related studies always introduced the use of this technique for some specific conditions, such as iatrogenic perforations in the colon or duodenum. Ryu *et al*[13] performed the purse-string suture technique to close iatrogenic colon perforations in eight cases and verified that EPSS can be appropriate for closing large colon perforations. In their analyses of the data obtained from 23 cases involving the closure of large iatrogenic duodenal perforations with purse-string sutures, Zhu *et al*[14] concluded that the EPSS method was feasible, effective, and easy for closure of perforations. Furthermore, some other studies focused on endoloop-assisted closure of exposed defects after EFTR

Table 4 Clinical features of 15 patients with adverse events

Age (yr)	Sex	Manipulation	Location	Size (cm)	Morphology	Closure type	Complete closure	Intraoperative perforation	Procedure time (min)	Delayed adverse events	Discharge (d)	Total cost (dollars)
60	Male	ESD	Fundus	3	Superficial SMT	C-EPSS	Yes	Yes	20	Delayed bleeding	10	2522
58	Male	EFTR	Fundus	4	Deep SMT	P-EPSS	Yes	Yes	15	Delayed perforation	11	2835
75	Female	ESD	Rectum	2.5	Superficial SMT	C-EPSS	No	No	22	Delayed bleeding	9	2710
56	Female	ESE	Fundus	3.5	Deep SMT	P-EPSS	No	No	24	Delayed perforation	6	2238
74	Female	ESD	Antrum	2	Precancerous lesion	C-EPSS	Yes	No	20	Delayed bleeding	7	2489
68	Male	ESD	Body	2.5	Deep SMT	C-EPSS	No	No	10	Delayed perforation	8	2470
58	Male	EFTR	Fundus	4	Superficial SMT	P-EPSS	Yes	Yes	15	Delayed perforation	7	2556
62	Male	ESE	Rectum	3.5	Precancerous lesion	C-EPSS	No	No	20	Delayed perforation	10	2884
63	Female	ESD	Antrum	3.5	Precancerous lesion	C-EPSS	No	No	12	Delayed bleeding	8	2690
71	Male	ESD	Rectum	2.5	Precancerous lesion	C-EPSS	Yes	No	14	Delayed perforation	6	2729
69	Male	ESE	Colon	2	Precancerous lesion	C-EPSS	Yes	No	18	Delayed perforation	8	2577
76	Male	EFTR	Fundus	3.5	Deep SMT	P-EPSS	Yes	Yes	25	Delayed perforation	8	2306
56	Female	ESD	Fundus	2.5	Superficial SMT	C-EPSS	Yes	Yes	10	Delayed bleeding	7	2423
61	Male	EFTR	Fundus	2.5	Deep SMT	C-EPSS	No	Yes	15	Delayed perforation	6	2789
70	Female	EFTR	Body	3.5	Superficial SMT	P-EPSS	Yes	Yes	20	Delayed bleeding	7	2687

SMT: Submucosal tumor; P-EPSS: Endoscopic pre-purse-string suture; C-EPSS: Conventional endoscopic purse-string suture; ESD: Endoscopic submucosal dissection; ESE: Endoscopic submucosal excavation; EFTR: Endoscopic full-thickness resection.

[35-39]. In comparison with other closure methods, the adoption in combination with endoclips may allow the management of larger post-EFTR defects and may reinforce the wound closure[24,40].

To the best of our knowledge, the present study is the largest case series exploring the effectiveness and safety of the EPSS method. A total of 180 cases involving EPSS-assisted closure performed at our medical center were enrolled in this study for further analysis. More importantly, our study was the first to compare the feasibility and efficacy of different closure types (P-EPSS and C-EPSS) used in endoscopic manipulations.

Table 5 Risk factors for delayed adverse events

Factors	Total (AE)	Odds ratio	95%CI	*P* value
Closure of defects				
Incomplete	11 (6)	21.33	5.45-83.45	< 0.01[1]
Complete	169 (9)	1		
Location (%)				
Fundus	79 (7)	1.13	0.39-3.26	0.82
Others	101 (8)	1		
Defect size				
≥ 3 cm	52 (8)	3.14	1.08-9.18	0.039[1]
< 3 cm	128 (7)	1		
Morphology				
Deep SMT	76 (6)	0.90	0.31-2.66	0.86
Others (superficial SMT and precancerous lesion)	104 (9)	1		
Closure type				
P-EPSS	63 (5)	0.92	0.30-2.83	1.00
C-EPSS	117 (10)	1		
Intraoperative perforation				
Yes	55 (7)	2.13	0.73-6.21	0.24
No	125 (8)	1		
Dissection method				
EFTR	36 (5)	2.16	0.69-6.78	0.19
Others (ESD and ESE)	144 (10)	1		

[1]Statistically significant.

SMT: Submucosal tumor; P-EPSS: Endoscopic pre-purse-string suture; C-EPSS: Conventional endoscopic purse-string suture; AE: Adverse event; ESD: Endoscopic submucosal dissection; ESE: Endoscopic submucosal excavation; EFTR: Endoscopic full-thickness resection.

P-EPSS has been employed as a novel closure method at our institution, accounting for 63 cases between 2014 and 2020. The previous study mainly used P-EPSS in the treatment of gastric tumors originating from the muscularis propria or gastric extra-luminal growth tumors[11]. In this study, we expanded the indications of P-EPSS and used this novel closure method in more situations, such as ESD or ESE cases with big defects. This approach is especially appropriate for defects characterized by a large size and specific positions or morphologies (*e.g.*, fundus or deep SMTs). All of the above clinical characteristics point to the same tendency, *i.e.*, intraoperative perforation. Thus, it is difficult and time-consuming to achieve complete closure, highlighting the need for careful selection of cases treated with P-EPSS-assisted closure.

In the present study, the P-EPSS group showed a significantly higher incidence of intraoperative perforation and a significantly shorter procedure time in comparison with the C-EPSS group. In this regard, the approach used to measure the procedure time of the EPSS method requires elucidation in advance. The time required for the resection procedure should be excluded for fairness, because the difficulty of endoscopic resection is different. Since the endoscopist had to take more time and effort to achieve complete closure of transmural defects, most of them would prefer to avoid the possibility of perforation in manipulations. Unexpectedly, the novel P-EPSS method could solve this problem from a different perspective by turning passive perforation to active perforation, and thereby providing sufficient time for preparation of perforation closure. Subtle and sensitive handling of the endoscope is essential for accomplishing the manipulation in EPSS. Although closure of the defects of transmural GI lesions revealed clinical effectiveness, this approach was not technically easy. The maneuverability of the endoscope was poor, especially in portions with big size and specific positions or morphologies. Further modifications are required to generalize this method in the future.

Regarding adverse events, the present study did not show that P-EPSS reduced the occurrence of adverse events, although the sample size may have been insufficient to detect differences between

groups because of the retrospective design. Interestingly, the selection of P-EPSS- or C-EPSS-assisted closure showed no relationship with the adverse event rate, but defects with incomplete closure and defect size larger than 3 cm were associated with worse outcomes. Cases of adverse events were successfully managed by endoscopic treatment and conservative therapy. After the treatment, the fasting period, duration of hospitalization, and total cost were similar between the two groups. Taken together, our results suggest that the P-EPSS procedure is an effective and safe method for closing the defects of difficult targets in endoscopic operations.

Our study had several limitations, including a selection bias caused by the inclusion of more cases of large defects and intraoperative perforation in the P-EPSS group; this was primarily attributable to the single-center retrospective design and should be taken into account when interpreting the findings. However, we could not eliminate selection bias. We tried to overcome these limitations by including consecutive patients during the study period. Second, all procedures were performed by expert endoscopists at a high-volume center, limiting the generalizability of the findings. We hope to design a prospective study with a different cohort to compare the criteria for choosing between P-EPSS or C-EPSS before the endoscopic manipulation. Nevertheless, despite these limitations, our study is the largest case series on the topic of EPSS-assisted endoscopic closure and the first report to present a comparison of P-EPSS and C-EPSS.

CONCLUSION

Our findings revealed that EPSS could achieve secure complete closure of mucosal defect. P-EPSS could shorten the procedure time and achieve secure complete closure of mucosal defects in clinical practice. Selection of P-EPSS was based on the defect's size, location, and the depth of the SMT. Defects with incomplete closure or size larger than 3 cm were associated with worse outcomes, rather than the selection of closure type.

ARTICLE HIGHLIGHTS

Research background

Endoscopic purse-string assisted suturing (EPSS) has proven to be an effective and safe technique for the closure of large mucosal defects. Recently, the endoscopic pre-purse-string suture (P-EPSS) procedure was invented and was widely used in clinical.

Research motivation

The novel invented P-EPSS should be analysed and compared with the conventional EPSS (C-EPSS) procedure.

Research objectives

Elucidate the outcomes of EPSS-assisted closure in different clinical situations, and evaluate the efficacy of P-EPSS.

Research methods

The study included a total of 180 patients who underwent closure assisted by EPSS between July 2014 and June 2020. The P-EPSS (n = 63) and C-EPSS (n = 117) groups were compared and the intergroup differences in aspects such as the lesion size, location, and morphology, incidence of complete closure, intraoperative perforation, and delayed adverse events were evaluated.

Research results

Patients with lesion size larger than 3 cm, lesions located at the fundus of stomach, or submucosal tumors originating from the deep mucosa were more likely to undergo P-EPSS-assisted closure. The P-EPSS group showed a significantly higher proportion of intraoperative perforation and a much shorter procedure time. But the incidence of adverse events did not differ significantly between P-EPSS and C-EPSS groups. Lesions with incomplete closure or size greater than 3 cm showed a statistical tendency to result in an increase in delayed adverse events.

Research conclusions

EPSS could achieve secure complete closure of mucosal defect. P-EPSS could shorten the procedure and yield complete closure of mucosal defects.

Research perspectives

To eliminate the selection bias, we would dedicate to design a new cohort prospective study to compare

the criteria of deciding in advance whether to use P-EPSS or C-EPSS before endoscopic manipulations.

FOOTNOTES

Author contributions: Li MM and Zhang Y contributed equally to this work; Li MM and Zhang Y drafted the manuscript and analyzed the data; Xu LM and Zhang Y designed the study and supervised its implementation; Xu LM, Zhang Y, Qu CY, and Shen F completed the endoscopic manipulations; Sun F, Huai MX, Zhang FY and Li ZH, participated in the experiments; and all authors made critical revisions and approved the final version to be published.

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ORIGINAL ARTICLE

Observational Study

Trends in gastrointestinal disease hospitalizations and outcomes during the first year of the coronavirus pandemic

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Abstract

BACKGROUND

The impact of the coronavirus on hospitalizations for gastrointestinal (GI) disease, particularly at a population level is understudied.

AIM

To investigate trends in hospitalizations, inpatient endoscopy resource utilization, and outcomes during the first year of the coronavirus pandemic and subsequent lockdowns.

METHODS

Using the California State Inpatient Database for 2018-2020, we explored year-to-year and 2020 month-to-month trends in hospitalizations, length of stay, and inpatient mortality (all-cause & viral pneumonia-specific) for common inpatient GI diagnoses including acute pancreatitis, diverticulitis, cholelithiasis, non-infectious gastroenteritis, upper and lower GI bleeding (LGIB), *Clostridium difficile*, viral gastroenteritis, inflammatory bowel disease, and acute cholangitis.

RESULTS

Disease-specific hospitalizations decreased for all included conditions except

nonvariceal upper GI bleeding (NVUGIB), LGIB, and ulcerative colitis (UC) (ptrend < 0.0001). All-cause inpatient mortality was higher in 2020 *vs* 2019, for acute pancreatitis (P = 0.029), diverticulitis (P = 0.04), NVUGIB (P = 0.003), and Crohn's disease (P = 0.004). In 2020, hospitalization rates were lowest in April, November, and December. There was no significant corresponding increase in inpatient mortality except in UC (ptrend = 0.048). Viral pneumonia and viral pneumonia complicated by respiratory failure increased (P < 0.001) among GI hospitalizations. Endoscopy utilization within 24 h of admission was unchanged for GI emergencies except NVUGIB (P < 0.001).

CONCLUSION

Our findings suggest that hospitalization rates for common GI conditions significantly declined in California during the COVID pandemic, particularly in April, November and December 2020. All-cause mortality was significantly higher among acute pancreatitis, diverticulitis, NVUGIB, and Crohn's disease hospitalizations. Emergency endoscopy rates were mostly comparable between 2020 and 2019.

Key Words: COVID-19; Shelter-in-place; Procedure utilization; Outcomes; Hospitalizations; Gastrointestinal diseases

Core Tip: In the current study, we found that, in the first year of the pandemic (2020), the lowest hospitalization rates for common gastrointestinal (GI) conditions in California coincided with peaks of the pandemic in April, November, and December. Overall, there was a 15% hospitalization rate reduction for acute GI conditions in 2020 (the first year of the pandemic) compared to 2019. No significant increase in all-cause mortality for GI admissions was observed for any of the conditions studied except acute pancreatitis, diverticulitis, nonvariceal upper GI bleeding (NVUGIB) and Crohn's disease. Emergency endoscopies within the first 24 h for acute GI conditions in 2020 were comparable with previous years except for NVUGIB. Unfortunately, there was a corresponding increase in all-cause mortality for NVUGIB. We also outline and create plots of the number of admissions and associated in patient all-cause mortality by month of the year for the conditions studied.

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INTRODUCTION

The COVID-19 pandemic had an immense impact on public health globally[1,2]. The first official case in the United States was confirmed on January 20, 2020[3]. Subsequently, containment measures were implemented with the goal of curtailing the spread of the coronavirus. On March 19th, 2020, the state of California became the first in the United States to issue a mandatory stay-at-home order[4]. This was eventually followed by other states which issued similar mandates[5]. In the wake of the spreading pandemic and implementation of control measures, there was a drastic decline in healthcare resource utilization for preventive, elective, and emergency purposes[2,6-8]. Widespread delays and outright refrainment from health care seeking were observed[9], as well as the dynamic restructuring of health systems to focus on expanding capacity for COVID response and limiting COVID exposure[7,8,10,11].

Multiple studies have revealed that at the peak of the pandemic, hospitalization rates among patients with acute gastrointestinal (GI) diseases dropped precipitously[12,13]. Additionally, widespread procedural delays and an overall decrease in the performance of endoscopic procedures were observed [14-16]. Many elective and ambulatory endoscopic procedures were canceled while health systems and national societies formulated guidelines. For instance, patients hospitalized with GI bleeding were found to have more severe laboratory parameters on presentation, lower odds of inpatient endoscopy, increased length of stay, and a higher likelihood of receiving a blood transfusion than those admitted during the pre-COVID period[17].

Diagnoses such as acute pancreatitis, diverticulitis, and GI hemorrhage have historically accounted for the majority of GI admissions in the United States[18]. While it is known that there was an overall

decline in admissions for non-COVID-related diagnoses, it remains unclear how outcomes of acute GI diseases evolved through the various waves of the pandemic and phases of lockdowns[7,19]. Existing data have emerged mostly from single-institution studies[7,9,15-17,20]. These studies have suggested an increase in emergency procedures during the pandemic but have yielded mixed results regarding outcomes such as in-hospital mortality rates[17,20-22].

In this study, we describe the trends in hospitalizations, endoscopy utilization, and inpatient mortality from 2018 to 2020 for twelve of the most common inpatient GI diagnoses, using a statewide database. Findings offer insight into how outcomes evolved through the various phases of the pandemic and provide population-level data beneficial to healthcare systems and policymakers.

MATERIALS AND METHODS

Data for this study was obtained from the California Healthcare Cost and Utilization Project State Inpatient Databases (HCUP-SIDs) for the years 2018 through 2020. The SID are a set of state-specific, all-payer, administrative databases maintained by the Agency for Healthcare Research and Quality as part of the HCUP. These data are collected annually and include discharge information from over 90% of discharges from eligible hospitals. The data elements captured include demographic information such as patient age, sex, race, marital status, zip code, payer, and income quartile (based on the median income for residential zip code) as well as elements pertaining to hospitalization such as length of stay, discharge disposition, primary and secondary discharge diagnoses, procedures, total hospital charges and outcomes[23,24]. An extensive description of the SID is available elsewhere[23,24]. Based on the determination that this study did not meet the Department of Health and Human Services definition of human subject research, this study was considered exempt by the Stanford University Institutional Review Board.

Study population

The analysis cohort comprised adult (≥ 18 years old) hospitalizations for 12 acute GI conditions in California, between January 1, 2018, and December 31, 2020. We used International Classification of Diseases, Tenth Revision, Clinical Modification (ICD-10-CM) codes, similar to previous studies (see appendix) for disease identification[25]. Hospitalizations for acute pancreatitis, cholelithiasis, diverticulitis, non-infectious gastroenteritis/colitis, non-variceal upper GI bleeding, variceal upper GI bleeding, lower GI bleeding/diverticular bleeding, *Clostridium difficile*, viral gastroenteritis, Crohn's disease, ulcerative colitis (UC) were included for analysis (see appendix for the ICD code algorithms used). These diagnosis categories were chosen based on previously published population-level studies that showed them as the most prevalent inpatient GI diseases per rank ordering[26]. Observations of 10 or fewer in count were excluded from reporting per the HCUP data use agreement. All authors had access to the study data and reviewed and approved the final manuscript.

Outcome measures

The outcomes of interest included year-to-year trends from 2018 to 2019, and 2020 month-to-month variations in acute GI hospitalizations, hospital length of stay, and all-cause inpatient mortality. We also explored viral pneumonia-associated mortality outcomes from 2018 to 2020 among patients hospitalized with GI conditions to assess the burden on these patients. In addition, we analyzed patterns in endoscopy intervention for emergency GI conditions such as food impaction, foreign body ingestion, acute variceal/non-variceal hemorrhage, and acute cholangitis during the pandemic. These procedures were defined using the International Classification of Diseases, Tenth Revision, procedural coding system (ICD-10 PCS) codes similar to those used in previous studies[26,27] (please see appendix for ICD codes used). Hospitalization costs were not estimated because at the time of analysis 2020 cost-to-charge data were not available. We followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines to improve the quality of reporting[28].

Statistical analysis

Descriptive statistics were used to describe demographic and clinical characteristics of GI-related hospitalizations. Frequencies and percentages were used to describe categorical variables. To compare categorical variables, we used Chi-square test. We compared demographic and clinical characteristics of GI admissions by year of hospitalizations. For selected GI conditions, we compared the total number of discharges, hospital length of stay, and in-hospital mortality by year. For selected GI procedures, we compared the total number of discharges, hospital length of stay, in-hospital mortality, number of procedures within the first 24 h of admission, and number of procedures after the first 24 h of admission. For estimating trends, we used logistic regression. Statistical significance was set at $P < 0.05$ and all tests were two-tailed. All analyses were performed using SAS version 9.4 (Cary NC, United States).

RESULTS

Baseline characteristics and hospitalization trends

Between January 1, 2018 and December 31, 2020, there were a total of 486543 hospitalizations for the 12 included acute GI conditions in California State. While annual hospitalizations were comparable between 2018 and 2019, there was a 15% hospitalization rate reduction in 2020 (173535 in 2018, 174827 in 2019, and 145545 in 2020). For all the years included in the analysis, acute cholelithiasis was the most common GI discharge diagnosis followed by acute upper GI bleeding, and then acute pancreatitis (Table 1). Disease-specific hospitalizations also significantly decreased for all conditions studied except non-variceal upper GI bleeding (NVUGIB) (P for trend = 0.853), lower GI bleeding (P for trend = 0.329), and UC (P for trend = 0.132) (Table 2). The 2020 month-to-month trend analysis revealed the lowest hospitalization rates in April, November, and December 2020, corresponding with the peaks of the pandemic (Figure 1A).

Baseline patient characteristics were similar across study years. Patients aged 45-64 years accounted for the greatest proportion of hospitalizations (33.2% in 2018, 32.7% in 2019, and 32% in 2020) while those aged ≥ 85 accounted for the smallest proportion (8.7% in 2018, 8.6% in 2019, and 8.3% in 2020) (Table 3). The majority of hospitalizations were among females (54% in 2018, 53.9% in 2019, and 52.7% in 2020) and Non-Hispanic Whites (48.3% in 2018, 46.8% in 2019, and 46.2% in 2020). Hispanics accounted for the second-highest proportion of hospitalizations (32% in 2018, 32.5% in 2019, and 33% in 2020) while other racial groups accounted for a minority of hospitalizations across all 3 study years (Table 3).

Mortality trends

For the entire cohort of GI hospitalizations, all-cause inpatient mortality was higher in 2020 compared to 2019 (1.2% *vs* 1.0%, $P < 0.001$). However, when individual diagnoses were examined, for most of the diseases, all-cause inpatient mortality was no different in 2020 compared to 2019 except among patients hospitalized with acute pancreatitis (0.6% *vs* 0.5%, $P = 0.029$), diverticulitis (0.6% *vs* 0.4%, $P = 0.04$), NVUGIB (2.1% *vs* 1.8%, $P = 0.003$), and Crohn's disease (2.2% *vs* 1.7%, $P = 0.004$) (Table 2).

To determine the trends in all-cause GI inpatient mortality, a 2020 month-to-month analysis was performed that did not reveal any significant variations except for UC hospitalizations where the highest inpatient mortality rate was observed in December 2020 (5%, P for trend = 0.048) (Table 2). We also examined month-to-month (Supplementary Figure 1), and year-to-year changes in viral pneumonia admissions among patients with the aforementioned GI diagnoses. We observed a significant increase in viral pneumonia hospitalizations among the entire cohort and also a significant increase in viral pneumonia-specific mortality from 2018 to 2020 (Table 3). In the absence of a dedicated ICD-10 code for COVID-19 in the years preceding the pandemic, we could not directly compare rates. We did however examine COVID-19-specific mortality rates for the year 2020 and found a mortality rate of 0.08%.

Endoscopy utilization

We examined patterns of endoscopy utilization including upper endoscopy, colonoscopy, flexible sigmoidoscopy, and endoscopic retrograde cholangiopancreatography (ERCP) in California between 2018 and 2020 for GI emergencies (Figure 1B). Except upper endoscopy for acute variceal hemorrhage which significantly reduced in 2020 (744 *vs* 690, $P = 0.007$), likely as a result of overall reduced hospitalization rates, there was no significant difference in inpatient endoscopic intervention rates in 2020 compared to 2019 (Table 4). Trend analysis showed that mortality for GI bleed emergencies was comparable for acute variceal and lower GI bleeding in the pre-pandemic and pandemic eras. However, in 2020, there was a significant rise in all-cause mortality among patients hospitalized with acute nonvariceal upper GI bleeding.

Sensitivity analyses demonstrated that for patients with acute nonvariceal bleeding, a significantly lower proportion received upper endoscopic intervention within the first 24 h of admission in 2020 compared to 2019 (30.3% *vs* 32.5%, $P < 0.001$), while the proportions were unchanged compared to previous years for patients hospitalized with acute variceal bleeding and lower GI bleeding (Table 4). ERCP utilization for acute cholangitis, intervention within the first 24 h (24.9% *vs* 27%, $P = 0.700$) and mortality from acute cholangitis (0.6% *vs* 0.4%. $P = 0.950$) did not significantly differ between all 3 years (Table 4). A graphical representation of monthly trends in endoscopy interventions and mortality in 2020 is shown in Supplementary Figure 2.

DISCUSSION

In this population-level study, we evaluated trends and outcomes of hospitalizations for acute GI conditions in 2020, the first year of the COVID pandemic in California, using a statewide database. Using the 2018 and 2019 data as a comparator group, we found significantly lower rates of hospitalization for most acute GI conditions in 2020 compared to previous years. We also found concomitant increases in all-cause inpatient mortality for four of the diagnoses studied including acute pancreatitis,

Table 1 Trends in discharges, hospital stay in days, and mortality for selected gastrointestinal diseases in California, 2018-2020

GI conditions	2018	2019	2020	Relative change	*P* for trend
Acute pancreatitis					
Total number of discharges, *n* (%)	26771 (0.8)	26675 (0.8)	23828 (0.8)	-11.0	0.028
Total hospital stays (in thousands) days	109.6	107.6	99.4	-9.3	0.118
Mortality, *n* (%)	124 (0.5)	134 (0.5)	144 (0.6)	16.1	0.029
Cholelithiasis					
Total number of discharges	39473 (1.2)	39710 (1.3)	33215 (1.2)	-15.9	< 0.001
Total hospital stays (in thousands) days	129.1	130.5	107.4	-16.8	0.152
Mortality, *n* (%)	96 (0.2)	94 (0.2)	81 (0.2)	-15.6	0.994
Diverticulitis					
Total number of discharges	16899 (0.5)	16992 (0.5)	13855 (0.5)	-18.0	< 0.001
Total hospital stays (in thousands) days	75.7	76.0	61.5	-18.8	0.495
Mortality, *n* (%)	77 (0.5)	67 (0.4)	87 (0.6)	13.0	0.042
Noninfectious gastroenteritis/colitis					
Total number of discharges	9376 (0.3)	9236 (0.3)	6734 (0.2)	-28.2	< 0.001
Total hospital stays (in thousands) days	27.3	27.8	21.3	-22.0	< 0.001
Mortality, *n* (%)	31 (0.3)	35 (0.4)	26 (0.4)	-16.1	0.543
Nonvariceal upper GI bleeding					
Total number of discharges	32207 (1.0)	33361 (1.1)	29155 (1.0)	-9.5	0.853
Total hospital stays (in thousands) days	125.6	132.6	116.9	-6.9	0.002
Mortality, *n* (%)	581 (1.8)	604 (1.8)	623 (2.1)	7.2	0.003
Variceal upper GI bleeding					
Total number of discharges	1007 (0.0)	880 (0.0)	808 (0.0)	-19.8	0.006
Total hospital stays (in thousands) days	4.0	3.7	3.2	-20.0	0.635
Mortality, *n* (%)	35 (3.5)	38 (4.3)	39 (4.8)	11.4	0.149
Lower GI bleeding and diverticular bleeding					
Total number of discharges	11549 (0.4)	11914 (0.4)	10613 (0.4)	-8.1	0.329
Total hospital stays (in thousands) days	43.4	44.2	40.0	-7.8	0.783
Mortality, *n* (%)	132 (1.1)	127 (1.1)	100 (0.9)	-24.2	0.145
Clostridium difficile					
Total number of discharges	1552 (0.0)	1457 (0.0)	963 (0.0)	-38.0	< 0.001
Total hospital stays (in thousands) days	9.8	9.3	6.3	-35.7	0.532
Mortality, *n* (%)	17 (1.1)	20 (1.4)	15 (1.6)	-11.8	0.308
Viral gastroenteritis					
Total number of discharges	6005 (0.2)	5652 (0.2)	3794 (0.1)	-36.8	< 0.001
Total hospital stays (in thousands) days	17.0	16.0	11.6	-31.8	< 0.001
Mortality, *n* (%)	15 (0.2)	19 (0.3)	18 (0.5)	20.0	0.066
Crohn's disease					
Total number of discharges	14273 (0.5)	14510 (0.5)	12583 (0.4)	-11.8	0.021
Total hospital stays (in thousands) days	76.6	77.5	70.1	-8.5	0.060
Mortality, *n* (%)	245 (1.7)	242 (1.7)	276 (2.2)	12.7	0.004
Ulcerative colitis					

Total number of discharges	12805 (0.4)	12919 (0.4)	11848 (0.4)	-7.5	0.132
Total hospital stays (in thousands) days	74.7	75.5	70.5	-5.6	0.361

GI: Gastrointestinal.

diverticulitis, NVUGIB, and Crohn's disease in 2020 compared to 2019. In addition, a 2020 month-to-month hospitalization trends analysis demonstrated the lowest hospitalization rates in April, November, and December of 2020.

The observed decline in hospitalizations for acute GI conditions is consistent with findings from previously published studies and likely reflects an interplay of factors during the COVID pandemic[12,13,29]. First, it has been suggested that people were willing to delay much-needed healthcare out of fear of potential exposure to COVID-19[9,11,29,30]. Additionally, the shelter-in-place measures in the spring of 2020 along with the restructuring of healthcare services necessitated by the pandemic are believed to have further reduced healthcare utilization for non-COVID indications[7,29].

The April nadir occurred after the implementation of multiple measures including the cancellation of all large events and the issuance of a stay-at-home order in March, by the California state government [13]. After the release of an initial reopening plan on April 28th, 2020 by the governor of California; and during the implementation of the early phases of the plan (in the summer months), we observed an uptick in hospitalization rates[31]. However, these never returned to pre-pandemic numbers. Coinciding with the reintroduction of restrictions later in the year, total hospitalization numbers for acute GI conditions plunged again. These lower hospitalization numbers continued through December as California and the rest of the United States battled another wave of the coronavirus pandemic[31].

Regarding GI outcomes with the fluctuations in hospitalization rates, fortunately, for most of the acute GI conditions studied, there was no significant increase in all-cause inpatient mortality, except for acute pancreatitis, diverticulitis, NVUGIB, and Crohn's disease admissions. Although we could not determine the time of symptom onset before admission, it is possible that previously reported delays in presentation coupled with higher acuity on presentation could have contributed to the observed increases in mortality for these conditions. A previous study of acute myocardial infarctions revealed significant delays in presentation and higher mortality in the setting of the pandemic[9]. Additionally, a collaborative report from Harvard T.H. Chan School of Public Health, the Robert Wood Johnson Foundation, and National Public Radio revealed that 2 in 10 adults had reported having household members who had delayed care for a serious medical condition in the setting of the pandemic. Furthermore, over 50% of those reporting delayed care had also reported experiencing resultant adverse effects[29]. The delayed presentation has been correlated with worse health outcomes[32].

Multiple studies reported increases in alcohol intake during the pandemic which could potentially explain the worse outcomes seen in acute pancreatitis hospitalizations[33,34]. However, we could not reliably ascertain the etiology of acute pancreatitis given the claims-based nature of the databases. In addition, we observed increased mortality among patients with Crohn's disease, but it remains unclear if the immunosuppression associated with the treatment of this inflammatory bowel disease increases the risk of COVID-related morbidity and mortality and/or if the excess mortality was attributable to COVID.

Although widespread procedural delays have been reported in the setting of the pandemic, our analysis of emergency procedural utilization revealed comparable rates of endoscopies for emergency GI conditions in 2020 with previous years, except when it came to NVUGIB where we observed a significant reduction in the proportion of patients receiving upper endoscopy within the first 24 h of presentation[14-16]. While this suggests that procedural delays could have played a role in the increase in NVUGIB-related inpatient mortality observed in 2020, it is difficult to attribute our findings solely to procedural delays as delays in presentation and higher acuity on presentation could have also played a role[9,29]. Nonetheless, current guidelines do recommend endoscopy within 24 h of presentation, and delays in endoscopy have been correlated with adverse outcomes in NVUGIB[35,36]. It is reassuring to note that outcomes of other GI emergencies such as acute variceal upper GI bleeding, lower GI bleeding, and acute cholangitis was not adversely impacted during the COVID pandemic. Although for these conditions, no reduction in emergency procedural utilization was noted.

The current study has multiple strengths. First, the use of a large, all-payer, statewide database allowed us to capture the impact of the pandemic at a large population level factoring in a diverse group of patients with different payer types. Additionally, our analyses of month-to-month trends for the year 2020, allowed us to evaluate trends in outcomes of interest in the light of the trajectory of the pandemic and our findings were reasonable as the lowest hospitalization numbers matched the phases of the documented lockdowns. We were also able to utilize ICD-10 codes and cohort identification algorithms that may reduce the risk of misclassification bias. Furthermore, we were able to compare 2020 data with the 2018 and 2019 SID data which provided baseline, pre-pandemic rates.

This study, however, has its limitations. First, given the widely varying approaches to the pandemic taken by individual states in the United States, generalizability might be limited. However, considering

Table 2 2020 month-to-month disease-specific mortality trends, *n* (%)

	January, 2020	February, 2020	March, 2020	April, 2020	May, 2020	June, 2020	July, 2020	August, 2020	September, 2020	October, 2020	November, 2020	December, 2020	Relative change	*P* for trend
Acute pancreatitis														
Total number of discharges	2163 (0.8)	1935 (0.8)	1956 (0.9)	1642 (0.9)	2002 (0.9)	2092 (0.9)	2063 (0.8)	2077 (0.9)	2106 (0.9)	2096 (0.8)	1913 (0.8)	1783 (0.7)	-17.6%	< 0.0001
Mortality	21 (1.0)	12 (0.6)	16 (0.8)	8 (0.5)	6 (0.3)	12 (0.6)	11 (0.5)	14 (0.7)	12 (0.6)	8 (0.4)	10 (0.5)	14 (0.8)	-33.3%	0.211
Cholelithiasis														
Total number of discharges	3170 (1.1)	2926 (1.1)	2437 (1.1)	2050 (1.1)	2743 (1.2)	2831 (1.2)	2759 (1.1)	2858 (1.2)	3112 (1.3)	3137 (1.3)	2864 (1.2)	2328 (1.0)	-26.6%	< 0.0001
Mortality	6 (0.2)	9 (0.3)	2 (0.1)	4 (0.2)	7 (0.3)	4 (0.1)	3 (0.1)	7 (0.2)	14 (0.4)	7 (0.2)	8 (0.3)	10 (0.4)	66.7%	0.060
Diverticulitis														
Total number of discharges	1349 (0.5)	1315 (0.5)	1067 (0.5)	746 (0.4)	1050 (0.5)	1141 (0.5)	1183 (0.5)	1302 (0.5)	1247 (0.5)	1351 (0.5)	1161 (0.5)	943 (0.4)	-30.1%	0.0004
Mortality	9 (0.7)	11 (0.8)	11 (1.0)	6 (0.8)	3 (0.3)	3 (0.3)	4 (0.3)	9 (0.7)	10 (0.8)	7 (0.5)	7(0.6)	7 (0.7)	-22.2%	0.501
Noninfectious gastroenteritis/colitis														
Total number of discharges	720 (0.3)	643 (0.3)	538 (0.2)	426 (0.2)	539 (0.2)	535 (0.2)	554 (0.2)	560 (0.2)	641 (0.3)	670 (0.3)	510 (0.2)	398 (0.2)	-44.7%	< 0.0001
Mortality	2 (0.3)	2 (0.3)	3 (0.6)	2 (0.5)	2 (0.4)	2 (0.4)	3 (0.5)	2 (0.4)	2 (0.3)	2 (0.3)	1 (0.2)	3 (0.8)	50.0%	0.859
Nonvariceal upper GI bleeding														
Total number of discharges	2971 (1.1)	2692 (1.1)	2458 (1.1)	1960 (1.0)	2361 (1.1)	2440 (1.0)	2451 (1.0)	2325 (1.0)	2428 (1.0)	2396 (1.0)	2450 (1.0)	2223 (0.9)	-25.2%	<.0001
Mortality	68 (2.3)	61 (2.3)	52 (2.1)	55 (2.8)	48 (2.0)	40 (1.6)	49 (2.0)	35 (1.5)	46 (1.9)	47 (2.0)	72 (2.9)	50 (2.2)	-26.5%	0.882
Variceal upper GI bleeding														
Total number of discharges	80 (0.0)	59 (0.0)	71 (0.0)	53 (0.0)	55 (0.0)	74 (0.0)	65 (0.0)	64 (0.0)	73 (0.0)	57 (0.0)	74 (0.0)	83 (0.0)	3.8%	0.042
Mortality	2 (2.5)	2 (3.4)	1 (1.4)	1 (1.9)	1 (1.8)	6 (8.1)	5 (7.7)	4 (6.3)	6 (8.2)	2 (3.5)	2 (2.7)	7 (8.4)	250.0%	0.050
Lower GI bleeding and diverticular bleeding														
Total number of discharges	1039 (0.4)	943 (0.4)	884 (0.4)	743 (0.4)	843 (0.4)	834 (0.4)	835 (0.3)	906 (0.4)	936 (0.4)	916 (0.4)	921 (0.4)	813 (0.3)	-21.8	0.231
Mortality	13 (1.3)	6 (0.6)	12 (1.4)	5 (0.7)	9 (1.1)	6 (0.7)	7 (0.8)	9 (1.0)	7 (0.7)	7 (0.8)	8 (0.9)	11 (1.4)	-15.4	0.822

Clostridium difficile														
Total number of discharges	96 (0.0)	98 (0.0)	85 (0.0)	63 (0.0)	67 (0.0)	81 (0.0)	82 (0.0)	80 (0.0)	89 (0.0)	95 (0.0)	70 (0.0)	57 (0.0)	-40.6	0.131
Mortality	2 (2.1)	2 (2.0)	4 (4.7)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.4)	4 (5.0)	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	-100.0	0.157
Viral gastroenteritis														
Total number of discharges	468 (0.2)	395 (0.2)	366 (0.2)	248 (0.1)	263 (0.1)	296 (0.1)	288 (0.1)	325 (0.1)	325 (0.1)	327 (0.1)	296 (0.1)	197 (0.1)	-57.9%	0.536
Mortality	4 (0.9)	0 (0.0)	2 (0.5)	2 (0.8)	2 (0.8)	3 (1.0)	0 (0.0)	0 (0.0)	2 (0.6)	1 (0.3)	2 (0.7)	0 (0.0)	-100.0%	0.402
Crohn's disease														
Total number of discharges	1262 (0.5)	1137 (0.4)	977 (0.4)	817 (0.4)	998 (0.5)	997 (0.4)	1076 (0.4)	1102 (0.5)	1120 (0.5)	1078 (0.4)	1040 (0.4)	979 (0.4)	-22.4%	0.035
Mortality	29 (2.3)	26 (2.3)	18 (1.8)	25 (3.1)	16 (1.6)	25 (2.5)	16 (1.5)	20 (1.8)	25 (2.2)	22 (2.0)	23 (2.2)	31 (3.2)	6.9%	0.627
Ulcerative colitis														
Total number of discharges	1109 (0.4)	1038 (0.4)	917 (0.4)	787 (0.4)	953 (0.4)	984 (0.4)	1039 (0.4)	973 (0.4)	1068 (0.4)	1081 (0.4)	970 (0.4)	929 (0.4)	-16.2%	0.0005
Mortality	31 (2.8)	32 (3.1)	19 (2.1)	28 (3.6)	19 (2.0)	23 (2.3)	22 (2.1)	23 (2.4)	26 (2.4)	27 (2.5)	35 (3.6)	46 (5.0)	48.4%	0.048
Acute cholangitis														
Total number of discharges	121 (0.0)	107 (0.0)	111 (0.0)	74 (0.0)	108 (0.0)	103 (0.0)	101 (0.0)	126 (0.1)	128 (0.1)	161 (0.1)	118 (0.0)	108 (0.0)	-10.7%	0.032
Mortality	1 (0.8)	0 (0.0)	0 (0.0)	2 (2.7)	0 (0.0)	1 (1.0)	1 (1.0)	0 (0.0)	1 (0.8)	1 (0.6)	0 (0.0)	2 (1.9)	100.0%	0.671

GI: Gastrointestinal.

that our study corroborates findings from previous studies, it is unlikely that the observed patterns are only limited to California[12,13]. Another limitation is that we could not explore time to presentation and therefore cannot definitively conclude that delays in presentation contributed to the observed increases in mortality. Our study also did not explore out-of-hospital mortality, making it possible that the overall pandemic-related excess mortality is higher than observed in our study.

Also, the timeframe of the data did not include 2021. Consequently, we were unable to explore the evolution of outcomes after the initiation of widespread vaccination, beginning in late 2020, as well as in the light of the Delta and Omicron variant-related surges. It is also not possible to ascertain if the trends continued into 2021. Finally, we have to emphasize the possibility of misclassification bias given our use of an administrative dataset.

Table 3 Demographic and clinical characteristics of select gastrointestinal hospitalizations in California, 2018-2020, *n* (%)

Variables	2018	2019	2020	*P* value
Age, yr				< 0.001
18-44	47019 (27.7)	46521 (27.2)	40924 (28.1)	
45-64	56369 (33.2)	5598 (32.7)	46629 (32.0)	
65-84	51757 (30.5)	53860 (31.5)	45944 (31.6)	
≥ 85	14693 (8.7)	14806 (8.6)	12062 (8.3)	
Sex				< 0.001
Male	78081 (46.0)	78928 (46.1)	68873 (47.3)	
Female	91745 (54.0)	92239 (53.9)	76677 (52.7)	
Race				< 0.001
White	81404 (48.3)	79531 (46.8)	66613 (46.2)	
African American	13080 (7.8)	13016 (7.7)	11219 (7.8)	
Hispanic	53962 (32.0)	55153 (32.5)	47629 (33.0)	
Asian Pacific Islander	13422 (8.0)	13894 (8.2)	11375 (7.9)	
Native American	555 (0.3)	835 (0.5)	485 (0.3)	
Other	6286 (3.7)	7336 (4.3)	6947 (4.8)	
Payer				< 0.001
Medicare	68639 (40.4)	70861 (41.4)	59701 (41.0)	
Medicaid	48581 (28.6)	48048 (28.1)	42046 (28.9)	
Private	45612 (26.9)	44389 (25.9)	37733 (25.9)	
Self-pay	3492 (2.1)	3864 (2.3)	2948 (2.0)	
No charge	204 (0.1)	272 (0.2)	170 (0.1)	
Other	3254 (1.9)	3707 (2.2)	2931 (2.0)	
Elixhauser comorbidity index				< 0.001
0	28927 (17.0)	28648 (16.7)	23244 (16.0)	
1	31228 (18.4)	31049 (18.1)	26001 (17.9)	
2	28036 (16.5)	28078 (16.4)	24118 (16.6)	
≥ 3	81647 (48.1)	83401 (48.7)	72196 (49.6)	
Bacterial pneumonia	4232 (2.5)	4285 (2.5)	3856 (2.6)	0.008
Bacterial pneumonia/respiratory failure specific mortality	248 (0.1)	271 (0.2)	275 (0.2)	0.009
Viral pneumonia	76 (0.0)	82 (0.0)	724 (0.5)	< 0.001
Viral pneumonia/respiratory failure specific mortality	6 (0.0)	5 (0.0)	80 (0.1)	< 0.001
COVID-19 specific mortality	-	-	123 (0.08)	< 0.001
All-cause mortality	1667 (1.0)	1644 (1.0)	1723 (1.2)	< 0.001

COVID-19: Coronavirus disease 2019.

CONCLUSION

In conclusion, our findings suggest that during the first year of the COVID pandemic, there was a significant decline in hospitalization rates for common GI conditions in California, particularly in the months of April, November, and December 2020. Reassuringly, 2020 emergency endoscopy rates were mostly comparable with 2019 rates except when it came to emergency endoscopy for NVUGIB. These findings suggest that inpatient healthcare delivery for most patients with acute GI conditions remained largely unchanged during the COVID-19 pandemic. They do however reveal that patients hospitalized with acute pancreatitis, diverticulitis, nonvariceal upper GI bleeding and Crohn's disease experienced

Table 4 Trends in discharges, mortality rate, and endoscopy utilization for gastrointestinal emergencies discharges in California, 2018-2020, *n* (%)

	2018	2019	2020	Relative change	*P* for trend
ANVH (upper endoscopy)					
Total number of discharges	15310 (0.5)	15942 (0.5)	13622 (0.5)	-11.0%	0.121
Total hospital stays (in thousands) days	65.0	69.2	60.2	-7.4%	0.002
Mortality	321 (2.1)	335 (2.1)	346 (2.5)	7.8%	0.012
Procedure within 24 h	5005 (32.7)	5185 (32.5)	4132 (30.3)	-17.4%	< 0.001
AVH (upper endoscopy)					
Total number of discharges	867 (0.0)	744 (0.0)	690 (0.0)	-20.4%	0.007
Total hospital stays (in thousands) days	3.6	3.2	2.8	-22.2%	0.697
Mortality	26 (3.0)	22 (3.0)	26 (3.8)	0.0%	0.414
Procedure within 24 h	348 (40.1)	327 (44.0)	283 (41.0)	-18.7%	0.653
LGIB (lower endoscopy)					
Total number of discharges	5524 (0.2)	5722 (0.2)	4973 (0.2)	-10.0%	0.724
Total hospital stays (in thousands) days	21.7	22.1	19.9	-8.3%	0.436
Mortality	29 (0.5)	35 (0.6)	17 (0.3)	-41.4%	0.201
Procedure within 24 h	859 (15.6)	975 (17.0)	793 (15.9)	-7.7%	0.534
Acute cholangitis (ERCP)					
Total number of discharges	1038 (0.0)	962 (0.0)	882 (0.0)	-15.0%	0.137
Total hospital stays (in thousands) days	4.3	4.4	3.7	-14.0%	0.505
Mortality	6 (0.6)	4 (0.4)	5 (0.6)	-16.7%	0.952
Procedure within 24 h	268 (25.8)	260 (27.0)	220 (24.9)	-17.9%	0.695
Food impaction (EGD/upper endoscopy)					
Total number of discharges	127 (0.0)	143 (0.0)	118 (0.0)	-7.1%	0.834
Total hospital stays (in thousands) days	0.6	0.6	0.6	0.0%	0.849
Mortality	4 (3.1)	3 (2.1)	0 (0.0)	-100.0%	0.086
Procedure within 24 h	73 (57.5)	70 (49.0)	60 (50.8)	-17.8%	0.289
Foreign body (EGD)					
Total number of discharges	21 (0.0)	17 (0.0)	20 (0.0)	-4.8%	0.891
Mortality	1 (4.8)	2 (11.8)	0 (0.0)	-100.0%	0.512
Procedure within 24 h	7 (33.3)	7 (41.2)	12 (60.0)	71.4%	0.091
SBP-paracentesis					
Total number of discharges	3254 (0.1)	3486 (0.1)	3344 (0.1)	2.8%	< 0.001
Total hospital stays (in thousands) days	32.4	35.3	33.8	4.3%	0.594
Mortality	621 (19.1)	631 (18.1)	592 (17.7)	-4.7%	0.147
Procedure within 24 h	928 (28.5)	1044 (30.0)	1022 (30.6)	10.1%	0.065

ANVH: Acute nonvariceal hemorrhage; AVH: Acute viral hepatitis; LGIB: Lower gastrointestinal bleeding; ERCP: Endoscopic retrograde cholangiopancreatography; EGD: Esophagogastroduodenoscopy; SBP: Spontaneous bacterial peritonitis.

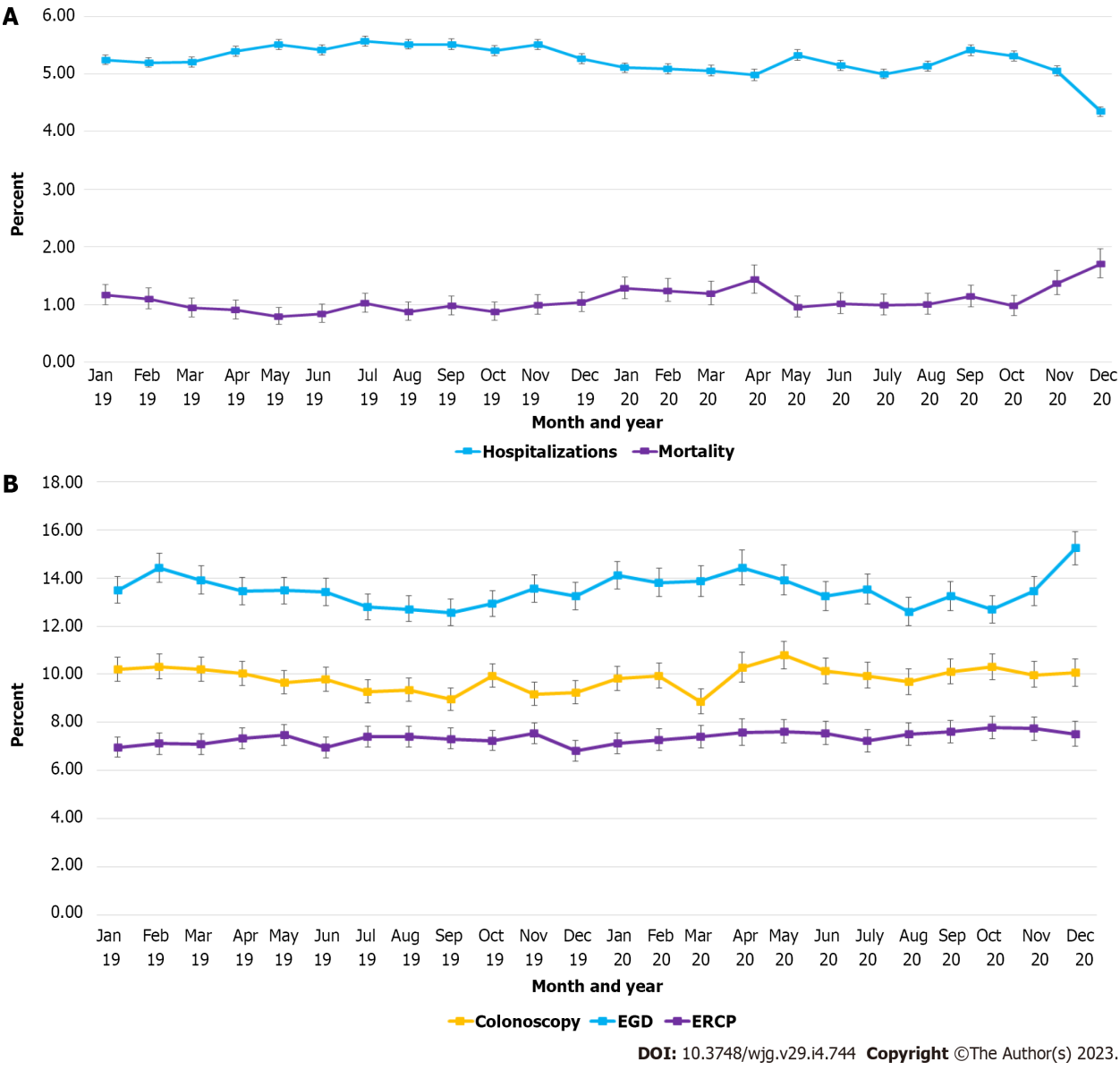

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Figure 1 Monthly trends. A: Monthly trends in hospitalization of selected gastrointestinal conditions and in-hospital mortality rates from January 2019 to December 2020; B: Monthly trends in endoscopy procedures among hospitalizations for selected gastrointestinal conditions from January 2019 to December 2020. EGD: Esophagogastroduodenoscopy; ERCP: Endoscopic retrograde cholangiopancreatography.

higher all-cause inpatient mortality during the pandemic and highlight that further research is needed to elucidate the disease-specific and system-based risk factors for the increase in mortality observed in these conditions.

ARTICLE HIGHLIGHTS

Research background

Healthcare resource utilization declined during the coronavirus pandemic. How this impacted gastrointestinal (GI) disease hospitalizations is not fully understood. We sought to investigate trends in hospitalizations, inpatient endoscopy utilization and outcomes during the first year of the pandemic and lockdowns.

Research motivation

The need for a population level understanding of the impact of the coronavirus pandemic on the outcomes of patients hospitalized with GI diseases.

Research objectives
To investigate trends in hospitalizations, inpatient endoscopy utilization and outcomes during the first year of the pandemic and lockdowns.

Research methods
Using the California State Inpatient Database for 2018-2020, we explored year-to-year and 2020 month-to-month trends in hospitalizations, length of stay, and inpatient mortality (all-cause & viral pneumonia-specific) for common inpatient GI diagnoses including acute pancreatitis, diverticulitis, cholelithiasis, noninfectious gastroenteritis, upper and lower GI bleeding (LGIB), *Clostridium difficile*, viral gastroenteritis, inflammatory bowel disease, and acute cholangitis, using regression analyses. We also investigated endoscopy utilization for GI emergencies.

Research results
Disease-specific hospitalizations decreased for all included conditions except nonvariceal upper GI bleeding (NVUGIB), LGIB, and ulcerative colitis (UC) (ptrend < 0.0001). All-cause inpatient mortality was higher in 2020 compared to 2019, for acute pancreatitis (P = 0.029), diverticulitis (P = 0.04), NVUGIB (P = 0.003), and Crohn's disease (P = 0.004). In 2020, hospitalization rates were lowest in April, November, and December. There was no significant corresponding increase in inpatient mortality except in UC (ptrend = 0.048). Endoscopy utilization within 24 h of admission was unchanged for GI emergencies except NVUGIB in which it was lower (P < 0.001).

Research conclusions
Our findings suggest that hospitalization rates for common GI conditions significantly declined in California during the COVID pandemic, particularly in April, November and December 2020. All-cause mortality was significantly higher among acute pancreatitis, diverticulitis, NVUGIB, and Crohn's disease hospitalizations. Emergency endoscopy rates were mostly comparable between 2020 and 2019.

Research perspectives
We observed that patients hospitalized with acute pancreatitis, diverticulitis, nonvariceal upper GI bleeding and Crohn's disease experienced higher all-cause inpatient mortality during the pandemic. Further research is needed to elucidate the disease-specific and system-based risk factors for the increase in mortality observed in these conditions.

FOOTNOTES

Author contributions: Okafor PN and Adekunle AD conceived and designed the study; Rubens M performed the statistical analysis; Adekunle AD, Rubens M, Sedarous M, and Okafor PN wrote and critically reviewed the manuscript; all authors reviewed and approved the manuscript.

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CASE REPORT

Pulmonary cryptococcosis after immunomodulator treatment in patients with Crohn's disease: Three case reports

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Abstract

BACKGROUND

Corticosteroids and anti-tumor necrosis factor α mAbs are widely used to treat Crohn's disease (CD). However, one disadvantage of this treatment is impairment of normal immune function, leading to an increased risk of infection. Cryptococcus infection is an opportunistic infection that occurs mainly in immunocompromised patients and poses a significant diagnostic challenge in patients with CD.

CASE SUMMARY

Here, we report three cases of pulmonary cryptococcosis in patients with CD after receiving immunomodulatory treatment. The patients presented with no or mild respiratory symptoms. Chest computed tomography scans revealed pulmonary nodules in the unilateral or bilateral lobes. Diagnoses were made using pathological examination and metagenomic sequencing. The patients were treated with fluconazole 400 mg once daily for 1 to 6 mo, and symptoms were resolved. Literature searches were conducted in PubMed, Web of Science, and Embase to retrieve previously reported cases and summarize patient characteristics.

CONCLUSION

The incidence of cryptococcus infection has increased along with immunomodulator use. Clinical vigilance is required for early identification and standardized treatment.

Key Words: Crohn's disease; Immunomodulator; Infliximab; Opportunistic infections; Pulmonary cryptococcosis; Case report

Core Tip: Corticosteroids and anti-tumor necrosis factor α mAbs are commonly used to treat Crohn's disease (CD). However, they may also contribute to an increased risk of opportunistic infections. In this article, we report three cases of pulmonary cryptococcosis in patients with CD after receiving immunomodulatory treatment. Pathogen identification mainly depends on pathological examination findings, but metagenomics can serve as an alternative tool. Patients with timely diagnosis generally have a good prognosis, but clinical alerts should be raised in those who are elderly and have comorbidities and dissemination phenotype.

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INTRODUCTION

Crohn's disease (CD), a chronic inflammatory disease that may involve any part of the digestive tract, is characterized by periodic clinical relapse and remission[1]. Treatment medications include mesalamine, corticosteroids, small-molecule immunosuppressants, and biologics such as mAbs[2]. However, these drugs also affect normal immune function and may contribute to an increased risk of opportunistic infections.

Pulmonary cryptococcosis is a type of subacute or chronic fungal infection caused by the genus *Cryptococcus*, and is found mostly in immunocompromised patients. It usually presents with isolated pulmonary granulomatous lesions but can also disseminate to other organ systems[3]. Here, we report three cases of pulmonary cryptococcosis in patients with CD who received immunomodulatory treatment, and review previously reported cases. Literature searches were conducted in PubMed, Web of Science and Embase databases using the keywords "Crohn's disease", "pulmonary cryptococcosis", and "cryptococcus". The search was restricted to full-text journal articles and conference abstracts published in English between January 1980 and May 2022. The bibliographies of the included articles were manually reviewed.

CASE PRESENTATION

Chief complaints

Case 1: A 65-year-old man was admitted to a local hospital for recurrent abdominal distension and pain 10 years ago.

Case 2: A 20-year-old man was admitted to our hospital for perianal pain with fever 5 years ago.

Case 3: A 59-year-old man was admitted to a local hospital for recurrent abdominal pain and diarrhea 13 years ago.

History of present illness

Case 1: The patient was referred to our hospital and diagnosed with CD. Before treatment initiation, chest computed tomography (CT) showed small nodules in the bilateral upper lobes with a benign tendency, pulmonary sac in the right upper lobe, and fibrous foci in the right middle lobe and bilateral lower lobes. The patient received four doses of infliximab (IFX; 300 mg) and achieved clinical remission.

Case 2: The patient was diagnosed with CD and was treated with IFX for 2 years (22 doses; 300 mg), which was discontinued due to secondary loss of response. He complained of night sweats and right-sided chest pain during deep breathing.

Case 3: The patient was diagnosed with CD 5 years ago and had been treated with methylprednisolone (MP; 0.75 mg/kg) for 3 years. He complained of productive cough for 1 mo.

Laboratory examinations

Case 2: The carbohydrate antigen 125 (CA-125) level was 54.2 U/mL. The G test, galactomannan (GM) test, and sputum fungal test results were negative.

Case 3: Vitamin B12 and folic acid levels were normal. Hepatitis B virus surface, core and E antibodies, antinuclear antibodies, vasculitis antibodies, sputum acid-fast (AF) staining, bronchoscopic brush

cytology, and bronchoalveolar lavage fluid (BALF) cytopathology showed negative findings.

Other laboratory examination results are summarized in Table 1.

Imaging examinations

Case 1: A repeat CT scan after the fourth dose of IFX revealed an increased number of bilateral nodules and a large nodule in the left upper lobe with possible inflammation (Fig. 1A). Electromagnetic navigation bronchoscopy revealed a clear trachea and no neoplasm in the principal and segmental bronchi. A biopsy was performed in the anterior segment of the left upper lobe. Fibrinobronchoscopic AF staining, fungus, culture, brush cytology, BALF Gram staining, exfoliative cytology, and X-PERT assay (simultaneous detection of *Mycobacterium tuberculosis* and resistance to rifampin) results were negative. Biopsy revealed focal granuloma formation in the alveolar tissues (Figure 1B). Periodic acid-Schiff (PAS) staining and AF staining were negative; however, periodic acid-silver methenamine (PASM) staining revealed suspicious positive bodies (Figure 1C).

Case 2: Chest CT revealed patchy lesions in the right upper lobe and right interlobar fissure, and multiple bilateral nodules with inflammatory propensity (Figure 2A). Bronchoscopy revealed clear segmental bronchi with no apparent stenosis or neoplasm in the lumen. Fibrinobronchoscopy was performed, and the BALF Aspergillus GM level was 0.840 μg/L. AF staining, fungus, brush cytology, and culture results were negative. BALF was sent for metagenomic sequencing and Cryptococcus was detected.

Case 3: Chest CT tomography revealed nodules in the posterior segment of the right upper lobe (Figure 3A). Tumor presence could not be ruled out. Bronchoscopy revealed no neoplasm in bilateral bronchi. Considering the difficulty of bronchoscopic biopsy, thoracoscopic lobectomy of the posterior segment of the right upper lobe was performed. Postoperative pathology revealed negative AF staining results but positive PAS and PASM staining results (Figure 3B and C). Tuberculosis- DNA (TB-DNA) test was negative.

FINAL DIAGNOSIS

Case 1-3: A diagnosis of CD combined with pulmonary cryptococcosis was confirmed.

TREATMENT

Case 1: IFX was discontinued, and the patient was administered fluconazole (FLCZ) 400 mg once daily for 6 mo.

Case 2: Medications for CD were discontinued. The patient was given FLCZ 400 mg once daily for 5 mo.

Case 3: MP was switched to pan-enteral nutrition therapy. The patient was administered FLCZ 400 mg once daily for 1 mo.

OUTCOME AND FOLLOW-UP

Case 1: A chest CT upon completion of anti-fungal therapy showed reduced size of the pulmonary nodule in the left upper lobe.

Case 2: During anti-fungal therapy, the patient was started on thalidomide for CD treatment and reported no apparent abdominal pain. One month after anti-fungal therapy, he achieved symptom resolution, and a repeat chest CT scan showed lesion absorption (Figure 2B).

Case 3: IFX was initiated 7 years later because of the progression of CD. Regular follow-ups have revealed no recurrence so far.

The clinical courses of the three cases are summarized in Figure 4.

DISCUSSION

Cryptococcus infection is an opportunistic fungal infection caused by *Cryptococcus neoformans* or *Cryptococcus gattii*. These pathogens are ubiquitously distributed, with the respiratory tract being the primary portal of entry. Cryptococcosis often occurs in immunocompromised individuals, such as human

Table 1 Laboratory examination

Case	1	2	3
WBC count (× 10^9/L)	10.0	9.4	2.5
Neutrophil count (× 10^9/L)	8.13	8.0	1.6
Platelet count (× 10^9/L)	305	282	220
Hemoglobin (g/L)	120	139	97
Albumin (g/L)	34.3	42.8	12.0
CRP (mg/L)	23.3	17.4	34.9
ESR (mm/hr)	19	16	14
Tumor markers	Negative	Negative	Negative
T-SPOT.TB	Negative	Negative	Weak positive
EBV IgM	Negative	Negative	-
Cytomegalovirus IgM	Negative	Negative	Negative
HIV	Negative	Negative	Negative
TPPA test	Negative	Negative	Negative

WBC: White blood cell; CRP: C reactive protein; ESR: Erythrocyte sedimentation rate; T-SPOT.TB: T-cell spot of tuberculosis test; EBV: Epstein-Barr virus; IgM: Immunoglobulin M; HIV: Human immunodeficiency virus; TPPA: Treponema pallidum particle agglutination.

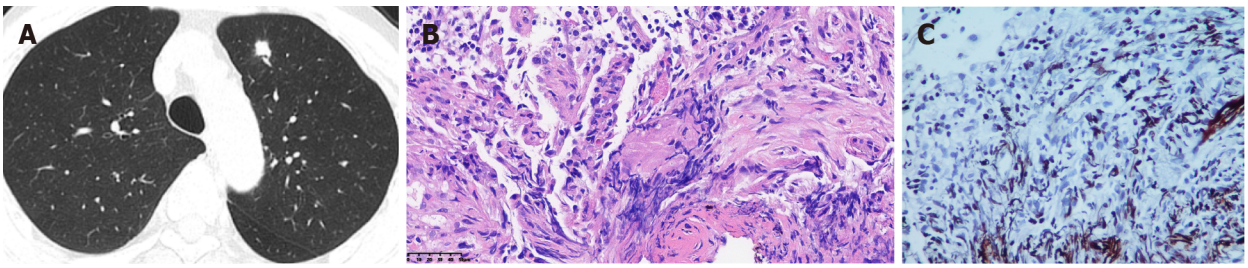

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Figure 1 Imaging and pathologic findings of case 1. A: Chest computed tomography scan showing multiple nodules in bilateral lobes and a large one in the left upper lobe; B: Hematoxylin-eosin staining showing focal granuloma formation in alveolar tissues; C: Periodic acid-silver methenamine staining showing suspicious positive organisms.

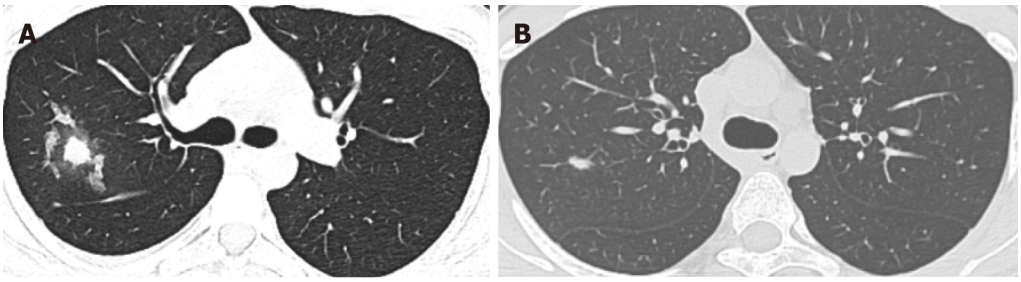

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Figure 2 Imaging findings of case 2. A: Chest computed tomography (CT) scan before fluconazole treatment showing patchy lesions in the right upper lobe and right interlobar fissure and multiple nodules in bilateral lobes; B: Chest CT scan showing lesion absorption 6 mo after initiation of anti-fungal therapy.

immunodeficiency virus-infected patients, organ transplant recipients, and those with indications for immunosuppressants[3]. However, it has also been reported in immunocompetent patients[4].

A total of 21 studies reporting CD cases complicated by Cryptococcus infection were identified (Table 2)[4-24]. Among them, one was a retrospective analysis[21] (197 males and 69 females) and another reported relapse of a previously reported case[13]. The remaining 19 patients comprised 13 males and 6 females; 9 of the cases were disseminated cryptococcosis[4,5,8,12,15-19]. Infection sites

Table 2 Clinical characteristics of cryptococcus infection in patients with Crohn's disease

Ref.	Age/sex	Comorbidity	CD medication	Symptom	Infection site	Treatment	Outcome
Lerner *et al*[5], 1988	65/M	Ankylosing spondylitis	Prednisone	Calf ulcer	Lungs, skin	AmB, 5FC	Died
Hrnicek *et al*[6], 2003	51/M	None	IFX, prednisone, MTX, ciprofloxacin	Cough, fatigue, headache, fever, chills	Lungs	Surgery, FLCZ	Recovered
Rehman *et al*[7], 2008	61/M	None	IFX, prednisone, budesonide, AZA	None	Lungs	AmB, 5FC → FLCZ	Recovered
Osawa *et al*, 2010 [8]	53/M	Silicosis	IFX, prednisone, AZA	Abdominal pain, diarrhea, fever, night sweats, headache	Lungs, GI, CNS	AmB → LAmB, 5FC → FLCZ	Recovered
Sciaudone *et al* [4], 2011	26/F	None	None	Diarrhea, abdominal pain, weight-loss, headache, cough	Lungs, GI	Surgery, LAmB → FLCZ	Recovered
Hirai *et al*[9], 2011	39/M	None	IFX	None	Lungs	Surgery	Recovered
Takazono *et al* [10], 2012	35/M	None	IFX, prednisolone, mesalazine	Fever	Lungs	FLCZ	Recovered
Fraison *et al*[11], 2013	54/M	Ankylosing spondylitis	ADM, AZA	Fever, anorexia, cough, chest pain, dyspnea, arthralgia, myalgia	Lungs	LAmB, 5FC → FLCZ	Recovered
Wysocki *et al* [12], 2015	46/M	None	IFX, AZA →ADM →CZP	Abdominal and back pain, fever, headache	Omentum, CNS	LAmB, 5FC	Recovered
Takazono *et al* [13], 2016	35/M	None	IFX, prednisolone	Fever, fatigue, night sweats	Lungs	FLCZ → ITCZ	Recovered
Zhou *et al*[14], 2016	66/M	Cryptogenic organizing pneumonia	Prednisone	Dyspnea, cough	Lungs	FLCZ	Recovered
Saad *et al*[15], 2016	71/M	None	IFX, AZA	Meningitis	CNS	-	-
Vasant *et al*[16], 2016	74/F	Posterior reversible encephalopathy syndrome	IFX, prednisolone	Headache, confusion, fever, rigor, body ache	CNS	LAmB, 5FC → VRCZ	Died
Lee *et al*[17], 2017	70/F	*Klebsiella pneumoniae* and *Pneumocystis jiroveci* infections	IFX	Pyrexia	CNS	AmB → VRCZ	Died
Chavapradit *et al* [18], 2018	64/F	None	Prednisolone, AZA, mesalazine	Abdominal pain, diarrhea	Lungs, GI	AmB → FLCZ	Recovered
Maleb *et al*[19], 2019	45/M	None	Steroid, AZA	Shock, abundance of ascites, fever	Pleural fluid, ascites	FLCZ	Died
Santo *et al*[20], 2019	23/M	Tuberculosis	IFX, AZA	Fever, headache	Lungs	FLCZ	Recovered
Yeh *et al*[22], 2021	57/F	Systemic lupus erythematosus	ADM, prednisolone, mesalazine	Pneumonia	Lungs	AmB, 5FC	Recovered
Hussein *et al* [23], 2021	54/M	None	IFX, prednisolone, MTX	Fever, fatigue, cough	Lungs	FLCZ	Recovered
Yeoh *et al*[24], 2022	52/F	Autoimmune hepatitis	Prednisolone	Dyspnea, chest pain, cough, lethargy	Lungs	Surgery	Recovered

M: Male; F: Female; IFX: Infliximab; MTX: Methotrexate; AZA: Azathioprine; ADM: Adalimumab; CZP: Certolizumab pegol; GI: Gastrointestinal tract; CNS: Central nervous system; AmB: Aamphotericin B; LAmB: Liposomal amphotericin B; 5FC: 5-fluorocytosine; FLCZ: Fluconazole; ITCZ: Itraconazole; VRCZ: Voriconazole; -: Not reported.

included lungs (15 cases), central nervous system (CNS; 5 cases), gastrointestinal tract (GI; 3 cases), skin (1 case), omentum (1 case), and pleural fluid and ascites (1 case).

Infected patients had been treated with different combinations of CD drugs, including anti-tumor necrosis factor α (TNF-α) agents, such as IFX, adalimumab and certolizumab pegol, corticosteroids, and other immunosuppressants. Symptoms ranged from asymptomatic lung granulomas to severe

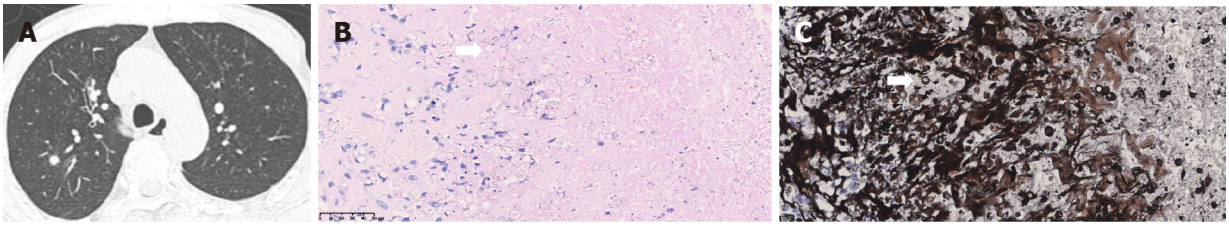

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Figure 3 Imaging and pathologic findings of case 3. A: Chest computed tomography scan showing nodules in the posterior segment of right upper lobe; B and C: Periodic acid-Schiff and periodic acid-silver methenamine staining showing positive organisms (white arrows).

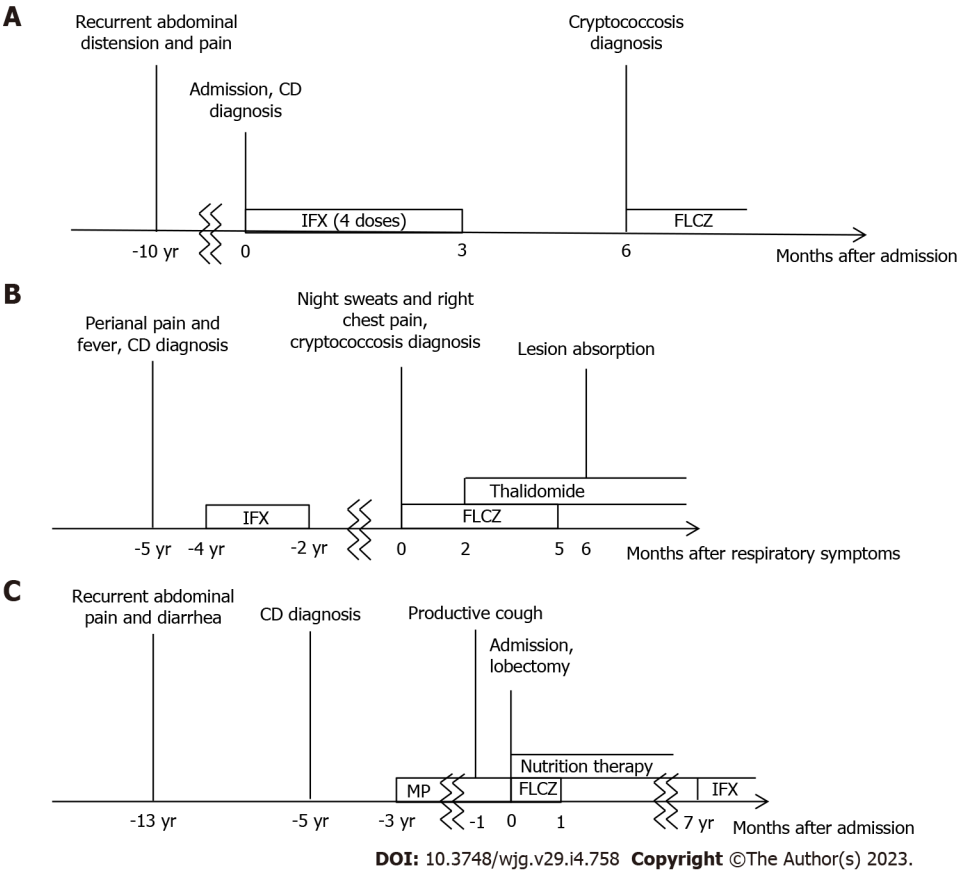

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Figure 4 Clinical courses of the three cases. A: Case 1; B: Case 2; C: Case 3. CD: Crohn's disease; IFX: Infliximab; FLCZ: Fluconazole; MP: Methylprednisolone.

pneumonia. Patients with disseminated cryptococcosis may also present with symptoms of other organ systems, such as colitis[8], meningitis[12], or skin ulcers[5]. Chest imaging typically revealed single or multiple pulmonary nodules. Diagnosis mainly relies on pathological examination, with accessory methods including fungal culture and the cryptococcal polysaccharide capsular antigen test. The most commonly used anti-fungal medications are amphotericin B (AmB), liposomal AmB (LAmB), 5-flucytosine (5FC), FLCZ, itraconazole, and voriconazole. Patients with mild symptoms generally recovered after treatment with triazole, while AmB and 5FC were intended for deep fungal infections; however, AmB and LAmB may cause serious adverse events[4,8]. Patients with disseminated cryptococcosis have a relatively poor prognosis, particularly when the CNS is involved.

Anti-TNF-α antibodies have been widely administered among CD patients with high efficacy, but concerns remain regarding their immunogenicity, skin toxicity, and increased risk of infection, such as the possible activation of latent TB. Therefore, current guidelines suggest that TB screening should be conducted before medication infusion[25]. TNF-α also plays a role in clearing pulmonary Cryptococcus infections by inducing interleukin-12 and interferon γ to promote a T1-cell-mediated immune response [26]. Anti-TNF-α agents, among other immunomodulators, may interfere with this process and increase the risk of *Cryptococcus* infection.

In this paper, we report three male patients diagnosed with CD, aged 20–65 years, who developed pulmonary cryptococcosis after being treated with anti-TNF-α agents or corticosteroids. The patients presented with asymptomatic or mild pneumonia with pulmonary nodules. One patient (case 2) showed elevated CA-125 level, which might be due to intestinal inflammation. G test and GM test were both negative, possibly because of low sensitivity of the tests for *Cryptococcus*. One patient (case 3) underwent surgery because of difficulty in biopsy. Diagnosis depended on postoperative pathological examinations since BALF might fail to detect *Cryptococcus*. All patients achieved symptom resolution after FLCZ treatment, consistent with other studies[6,10,23].

CD complicated with pulmonary cryptococcosis is relatively rare, but its incidence has increased in recent years with the use of various immunomodulators, particularly biologics, in CD management. Diagnosis is challenging because clinical symptoms and chest imaging both lack specificity, and GI manifestations can resemble CD progression, leading to misdiagnosis. Therefore, high clinical vigilance is required for early identification of infection, discontinuation of immunomodulators, and standardized treatment.

CONCLUSION

Our three case reports and literature review suggest that patients with timely diagnosis generally have a good prognosis. However, comorbidities, advanced age, and dissemination phenotype should raise clinical alerts. Metagenomic sequencing can be an alternative approach for pathogen diagnosis when biopsy is unfeasible.

FOOTNOTES

Author contributions: Fang YF and Cao Q contributed to conceptualization; Fang YF and Cao XH contributed to data collection and manuscript drafting; Fang YF, Cao XH and Yao LY contributed to manuscript revision; All authors have approved the final manuscript.

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