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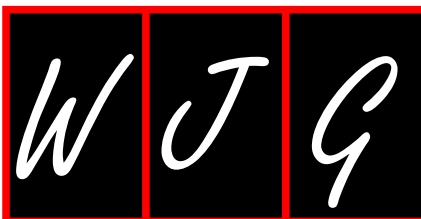
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Segmental colitis associated diverticulosis syndrome

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Abstract

Segmental colitis associated diverticulosis (SCAD) has become increasingly appreciated as a form of inflammatory disease of the colon. Several features suggest that SCAD is a distinct disorder. SCAD tends to develop almost exclusively in older adults, predominantly, but not exclusively, males. The inflammatory

process occurs mainly in the sigmoid colon, and usually remains localized to this region of the colon alone. SCAD most often presents with rectal bleeding and subsequent endoscopic visualization reveals a well localized process with non-specific histopathologic inflammatory changes. Granulomas are not seen, and if present, may be helpful in definition of other disorders such as Crohn's disease of the colon, an entity often confused with SCAD. Bacteriologic and parasitic studies for an infectious agent are negative. Normal rectal mucosa (*i.e.*, "rectal sparing") is present and can be confirmed with normal rectal biopsies. SCAD often resolves spontaneously without treatment, or completely after a limited course of therapy with only a 5-aminosalicylate. Recurrent episodes may occur, but most often, patients with this disorder have an entirely self-limited clinical course. Occasionally, treatment with other agents, including corticosteroids, or surgical resection has been required.

Key words: Segmental colitis associated diverticulosis syndrome; Ulcerative colitis; Diverticulitis; Segmental colitis; Diverticulosis; Inflammatory bowel disease

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Core tip: Segmental colitis associated diverticulosis is an increasingly recognized inflammatory disorder involving the colon, particularly the sigmoid colon. The disorder occurs mainly in males, often presents with rectal bleeding, and endoscopic evaluation usually reveals a localized non-granulomatous process in the sigmoid colon, frequently with rectal sparing. The clinical course is generally self-limited, sometimes resolving spontaneously or responding to minimal treatment.

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INTRODUCTION

Segmental involvement of the colon with a localized inflammatory process was noted in early clinicopathological descriptions following recognition that pathological features attributed to Crohn's disease of the colon could be distinguished from those of ulcerative colitis^[1-3]. Even in some of these, however, diverticular disease, particularly in the sigmoid colon was noted. In some, Crohn's disease was considered to be a co-existent inflammatory process with sigmoid diverticulosis, particularly in elderly patients^[4]. In contrast, others believed that this form of segmental colitis with diverticulosis was a distinct form of inflammatory bowel disease^[5]. It usually occurred in the elderly with sigmoid diverticulosis, but failed to demonstrate other associated features of Crohn's disease. In addition, this form of inflammatory process often appeared to respond to drug therapy and usually had a benign clinical course^[6,7].

SEGMENTAL COLITIS ASSOCIATED DIVERTICULOSIS SYNDROME

In recent years, segmental colitis associated with diverticulosis, or segmental colitis associated diverticulosis (SCAD), has further become recognized as a distinct clinical and pathological entity. Most cases occur in males, usually following initial presentation with rectal bleeding (over 70%). Usually, the entity is almost exclusively a disorder of the elderly, often after age 50 years, and in some, referral is often made to exclude an occult colorectal malignancy^[8]. In some, diarrhea and/or abdominal pain were also present. These clinical features substantially differed from the predominately female sex distribution and younger age of patients initially diagnosed with Crohn's disease^[9] as well as in the occasionally encountered patient with Crohn's disease diagnosed after age 60 years^[10], both long-term studies from the same center. A prominent clinical feature, even in long-term follow-up studies, is responsiveness to limited treatment, often only an oral 5-aminosalicylate (5-ASA). In some, spontaneous resolution and remission without medication has been noted, even in those followed for several years. Sometimes, however, persistent chronically active and symptomatic disease developed, or recurrent separate episodes, all leading to use of corticosteroids and/or eventual surgical resection, usually of the involved sigmoid colon segment.

PATHOLOGICAL AND LABORATORY FEATURES

SCAD is pathologically defined by a non-specific segmental or localized non-granulomatous inflammatory process, usually confined to the sigmoid colon. Rectal sparing is best documented by direct macroscopic

visualization and histopathologic confirmation of normal mucosa in the rectum. Multiple diverticula are also clearly seen. An endoscopic feature of SCAD is that inflammation is detected within the inter-diverticular mucosa without necessarily involving diverticular orifices^[11] or as so-called crescentic fold disease^[12]. Similar localized changes have been reported recently with ipilimumab-associated colitis^[13]. Fever and leukocytosis are characteristically absent^[14]. Most routine laboratory studies are normal, including fecal studies for bacterial and parasitic agents. Serological markers, specifically perinuclear neutrophil cytoplasmic antibodies (*i.e.*, pANCA) and anti-Saccharomyces cerevisiae antibodies (*i.e.*, ASCA), often positive in other forms of inflammatory bowel disease including ulcerative colitis and Crohn's disease^[15], have been routinely negative^[8], while fecal calprotectin may be useful in differentiating SCAD from other healthy controls or the irritable bowel syndrome^[16].

LONG-TERM STUDIES

In a long-term study over a 20-year period^[8], over 80% (*i.e.*, 21 of 24) received oral 5-ASA (*i.e.*) medication while the others elected not to receive treatment. No patient was treated with oral or intravenous antibiotics. All subsequently had a complete clinical and pathological resolution, including those that were not treated. Over 80% (*i.e.*, 17 of 21) treated with 5-ASA developed a remission within 6 mo, but some had persistent symptoms for more than a year, and in one, recurrent episodes of pain occurred for 7 years. Some were non-compliant with prescribed 5-ASA, however, 2 eventually resolved completely while 3 others were administered added prednisone. A total of 3 eventually required an anterior resection for persistent sigmoid inflammatory stricture.

NATURAL HISTORY OF SCAD

The natural history of SCAD has also been explored^[8]. Although recurrent SCAD was documented over a year after complete clinical and pathological resolution of the first episode, over 60% of patients suffering recurrent SCAD developed their second episode of disease more than a decade after the initial clinical episode. This study group was of particular interest because of the absence of sigmoid colonic neoplasia, given the older age of this patient group. In 3, endoscopic removal of colonic adenomas had been completed before clinical presentation with rectal bleeding and SCAD detection, while 1 had an adenoma resected after diagnosis of SCAD. Two others also developed a colon cancer, one in the rectum 4 years after SCAD had resolved, and one in the cecum 9 years after SCAD had resolved. In the latter, a resection was done for an early stage lesion. Interestingly, recurrent SCAD developed 5 years later and resolved within 2 mo using 5-ASA alone. Others have also emphasized the benign nature of this

segmental inflammatory process in a similar 7-year follow-up study^[17].

CONCLUSION

This entity, SCAD, also should have a very special clinical relevance^[18] to physicians caring for patients with inflammatory bowel disease. First, long-term studies indicate that the disease appears to often be a self-limited inflammatory process that resolves without any future disease episode or requirement for ongoing treatment. Clearly, there are some similarities of SCAD to other forms of inflammatory bowel disease, particularly Crohn's colitis. Indeed, in a Dutch study, retrospective evaluation of multiple biopsies and further appreciation for the disease course resulted in definition of SCAD in an estimated 8% of cases, particularly if diverticulosis was present^[19]. As such, the implications of an inaccurate diagnosis should be clearly obvious. A case of SCAD in the sigmoid colon labeled as Crohn's disease could conceivably lead the treating physician or clinical trial investigator (in the case of new forms of therapy) to conclude that a positive outcome was treatment-related rather than related to the natural history of an otherwise benign process. Second, although some inflammation-associated changes in SCAD may be shared with other forms of inflammatory bowel disease and its treatment, including expression of tumor necrosis factor alpha and its downregulation with treatment^[20], definition of distinct or "new" inflammatory process that has the ability to resolve with complete mucosal healing suggests a critically important need to further explore the molecular events in SCAD, especially if these could lead to complete resolution or "cure" of other more commonly recognized inflammatory processes in the intestine.

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Guanylyl cyclase C signaling axis and colon cancer prevention

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Abstract

Colorectal cancer (CRC) is a major cause of cancer-related mortality and morbidity worldwide. While improved treatments have enhanced overall patient outcome, disease burden encompassing quality of life, cost of care, and patient survival has seen little benefit. Consequently, additional advances in CRC treatments remain important, with an emphasis on preventative measures. Guanylyl cyclase C (GUCY2C), a transmembrane receptor expressed on intestinal epithelial cells, plays an important role in orchestrating intestinal homeostatic mechanisms. These effects are mediated by the endogenous hormones guanylin (GUCA2A) and uroguanylin (GUCA2B), which bind and activate GUCY2C to regulate proliferation, metabolism and barrier function in intestine. Recent studies have demonstrated a link between GUCY2C silencing and intestinal dysfunction, including tumorigenesis. Indeed, GUCY2C silencing by the near universal loss of its paracrine hormone ligands increases colon cancer susceptibility in animals and humans. GUCY2C's role as a tumor suppressor has opened the door to a new paradigm for CRC prevention by hormone replacement therapy using synthetic hormone analogs, such as the FDA-approved oral GUCY2C ligand linacotide (LinzessTM). Here we review the known contributions of the GUCY2C signaling axis to CRC, and relate them to a novel clinical strategy targeting tumor chemoprevention.

Key words: Colorectal cancer; Guanylin; Uroguanylin; Chemoprevention; Heat-stable enterotoxins; Cyclic guanosine monophosphate; Guanylyl cyclase C

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Core tip: Guanylyl cyclase C (GUCY2C) is a tissue-specific transmembrane receptor found in apical membranes of intestinal enterocytes that drives intestinal homeostatic mechanisms and opposes colorectal tumorigenesis. The receptor's endogenous hormone ligands, guanylin and uroguanylin, are the most commonly lost gene products in colorectal cancer, making GUCY2C a potential therapeutic target for tumor prevention through hormone replacement therapy.

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INTRODUCTION

Colorectal cancer (CRC) is the fourth most common cancer in the United States and the second-leading cause of cancer-associated death^[1,2]. It is estimated that approximately one million new cases of CRC will occur in 2016, and approximately 600000 people will succumb to the disease worldwide^[2,3]. CRC incidence and mortality rates have experienced a continuous decline over the past few decades, specifically due to marginal improvements in treatment^[4], awareness of CRC risk-factors^[3], and routine early detection screening^[5]. However, the high prevalence of adenomatous polyp diagnoses despite the growth in screening awareness have led to persistently high morbidity and mortality rates for CRC, demonstrating the continual clinical need for improved CRC prevention and treatment strategies. Currently, CRC prognosis is dependent upon disease stage, with primary treatment for localized disease resulting in complete surgical resection of the tumor. Although surgery has contributed to a 5 year survival rate of greater than 65%, disease reoccurrence has been reported in nearly 50% of CRC patients^[6,7]. With nearly one fourth of patients presenting with metastatic disease, it is crucial to develop highly effective therapies targeted towards earlier prevention and treatment that slows disease initiation and progression^[4,8].

Colorectal tumorigenesis is widely accepted as a disease of both sporadic and inherited genomic instability resulting in three forms of CRC: (1) microsatellite instability (MSI); (2) chromosomal instability [CIN; *i.e.*, the activation of oncogenes (K-ras) and inactivation of tumor suppressors (*adenomatous*

polyposis coli, APC; p53)]; and (3) chromosomal translocations^[9-12]. Apart from oncogenomic risk factors leading to CRC, recent work suggests that loss of the guanylyl cyclase C (GUCY2C) signaling cascade contributes to CRC susceptibility^[13-15]. In an effect preserved across species, CRC is associated with a near universal loss of the GUCY2C hormone ligands guanylin and uroguanylin, disrupting intestinal homeostasis and initiating disease progression^[14,16-21]. While the processes by which these hormone ligands are suppressed in CRC remain unknown, they are of great interest to better understand mechanisms of tumorigenesis. This emerging role of GUCY2C as a tumor suppressor in the intestine, whose loss of signaling is the result of a hormone deficiency, provides a unique opportunity to reactivate GUCY2C signaling through hormone replacement therapy^[15]. This review highlights recent insights in the GUCY2C-hormone signaling axis, its relation to the previously defined hallmarks of CRC, and its potential as a preventative treatment option.

GENETIC PATHWAYS FOR CRC

Sporadic CRC arises predominantly through a characteristic accumulation of acquired somatic mutations over decades, leading to cellular transformation, subsequent adenoma formation, and ultimately malignancy^[9,22,23]. Hereditary CRC, despite comprising a only 15%-30% of all CRC cases, have helped provide insight into this stepwise accumulation of mutations implicated in the pathogenesis of sporadic CRC^[24].

Almost all inherited colorectal syndromes are categorized either as familial adenomatous polyposis (FAP) or hereditary nonpolyposis CRC (HNPCC)^[25]. In FAP, individuals possess an inactivating mutation in the *adenomatous polyposis coli* (APC) gene. Over time, sporadic mutations to the remaining APC gene result in cells with complete loss of APC function. These cells then expand to generate adenomas, some of which then progress to malignant adenocarcinoma. The APC protein functions as an essential regulatory element in the canonical Wnt signaling pathway, preventing the accumulation of oncogenic β -catenin by promoting its degradation. In the absence of functional APC, β -catenin accumulates and translocates to the nucleus, where it acts as a cofactor for the oncogenic heterodimer transcription factor TCF/LEF^[26]. This heterodimer increases transcription of genes involved in cell proliferation and growth, including the proto-oncogenes c-MYC and CCND1 (cyclin D1)^[26]. The loss of functional APC is a key event for adenoma initiation, as it occurs early in the morphogenesis model of CRC^[22].

Alternatively, HNPCC arises from mutations to a protein complex responsible for DNA mismatch repair (MMR)^[24,27]. This complex, which is conserved across biological kingdoms, recognizes and binds mismatched DNA, then excises and repairs the error. Mutations

in MMR genes, like *MSH2* and *MLH1*, increase the accumulation of additional DNA mutations, leading to increased genomic instability, particularly in microsatellites^[27]. Although mutations in MMR genes are relatively uncommon in sporadic CRC, MSI still occurs in approximately 10%-15% of cases, indicating that genomic instability and inadequate DNA repair play important roles in CRC development^[25,27].

INTESTINAL HOMEOSTATIC MAINTENANCE BY THE GUCY2C SIGNALING AXIS

GUCY2C is one of several mammalian transmembrane guanylyl cyclase receptors that react to extracellular signals present in the microenvironment^[16,28]. GUCY2C is predominantly expressed on apical brush border membranes of intestinal enterocytes, transducing extracellular peptide signals in the gut lumen into intracellular signaling cascades required for normal intestinal physiology^[16,29]. GUCY2C was originally identified as an orphan receptor for the bacterial heat-stable enterotoxin, STa, produced by a number of enteric bacteria including enterotoxigenic *Escherichia coli* (ETEC)^[30]. STa functions as a GUCY2C agonist, inducing a signaling cascade that causes excessive fluid and electrolyte secretion into the intestinal lumen, which manifests clinically as enterotoxigenic "traveler's" diarrhea^[31]. Interaction of STa with the GUCY2C extracellular ligand-binding domain activates its cytoplasmic catalytic domain, driving the conversion of GTP to cyclic guanosine monophosphate (cGMP)^[16,28,29]. Intracellular cGMP then operates as a second messenger for downstream signaling, specifically activating cGMP-dependent protein kinase II, which then phosphorylates and activates the cystic fibrosis conductance regulator (CFTR). Activation of CFTR induces chloride secretion into the intestinal lumen, generating an electrochemical gradient that drives sodium into the gut lumen. Combined with cGMP-induced inhibition of the sodium-hydrogen exchanger (NHE3), CFTR activation elevates extracellular solute concentration to generate an osmotic gradient resulting in fluid accumulation in the lumen^[16,28,29].

To date, two novel mutations in GUCY2C that affect gastrointestinal motility have been identified. The first, an autosomal dominant "gain of function" mutation in a Norwegian family, reflected a non-synonymous mutation resulting in the substitution of serine for isoleucine at residue 840 of the GUCY2C catalytic domain. This mutation increased ligand-dependent cGMP production which manifested clinically as chronic diarrhea and increased susceptibility to inflammatory bowel disease (IBD)^[28,32,33]. Separately, two autosomal recessive inactivating GUCY2C mutations were discovered in two Bedouin families which reduced GUCY2C function leading to neonatal meconium ileus^[28,34].

Exogenous STa is a molecular mimic of two

endogenous peptide ligands, which also function as GUCY2C agonists. These ligands, guanylin (GUCA2A) and uroguanylin (GUCA2B), both expressed in gut epithelial cells^[15,35,36], act locally as autocrine and paracrine hormones to regulate GUCY2C signaling and fluid and electrolyte homeostasis^[28,31]. Additionally, uroguanylin acts as an endocrine hormone, secreted into the systemic circulation postprandially to activate hypothalamic GUCY2C and induce satiety^[37-39]. Although GUCY2C signaling is utilized by bacteria to induce pathogenic diarrhea, several important characteristics differentiate endogenous guanylin and uroguanylin from exogenous STa. First, guanylin and uroguanylin have 10- to 100-fold lower affinities for GUCY2C than STa. Further, unlike STa, which contains three disulfide bonds, guanylin and uroguanylin contain only two disulfide bonds, increasing their susceptibility to proteolytic degradation in the gut lumen in comparison to STs^[16,35].

The cellular sources of these intestinal peptides in both rodents and humans have been explored. Seminal studies utilizing custom antibodies described guanylin protein expression as confined to mature goblet cells throughout the rat small intestine and colon, as well as the columnar epithelial cells of the colon^[40]. These data were supported by Brenna *et al.*^[41], which utilized *in situ* hybridization to identify guanylin mRNA expression in rat and human goblet cells and colonocytes. Guanylin mRNA also was enriched in both the rat and human duodenum, however cell-specific guanylin expression differed between species^[41]. Immunohistochemistry first identified uroguanylin protein expression in rat proximal small intestine, with enrichment in enterochromaffin cells (EC)^[42]. In contrast, *in situ* hybridization experiments by Brenna *et al.*^[41] did not detect uroguanylin mRNA expression in cells co-expressing CHGA, a marker for EC, in either rat or human intestine. Although this study further supported localized expression of uroguanylin in rat and human proximal small intestine, specifically the duodenum, the precise cellular origin of uroguanylin remains incompletely defined.

GUCY2C SIGNALING AXIS AND CRC

In addition to its role in the regulation of fluid and electrolyte balance, GUCY2C also serves a protective role against colorectal tumorigenesis^[43,44]. The mammalian intestinal tract is lined with differentiated columnar cells derived from progenitor cells that differentiate during their progression along the crypt-villus unit^[45,46]. The inner lining of the intestine is one of the most rapidly renewing tissues in the human body, with the entire epithelial surface turning over every 4-5 d^[47]. Consequently, the intestine must coordinate a delicate balance between cell proliferation, differentiation, migration and apoptosis to prevent tumorigenesis^[46-48]. GUCY2C, expressed in all cells lining the small intestine and colon, helps maintain this delicate balance. In mice

lacking GUCY2C (*Gucy2c*^{-/-}) this disrupted homeostasis manifests as an increase in proliferative cells, poorly developed differentiated compartments, accelerated cell cycle, and increased crypt depth, ultimately increasing the risk of tumorigenesis^[31].

These observations suggest that events that impair GUCY2C signaling, such as loss of receptor or hormone expression, should similarly increase the risk of tumorigenesis, and this is indeed the case. In that context, several conditions associated with CRC, such as IBD^[32,49,50] and obesity, silence GUCY2C signaling prior to overt tumorigenesis, re-enforcing the possibility that silenced GUCY2C mediates the pathogenesis of tumor initiation and progression^[51,52]. Importantly, in both of these cases, GUCY2C silencing is mediated by impaired ligand expression rather than loss of GUCY2C, providing a potential therapeutic target in CRC. Conversely, epidemiological observations revealed that areas of endemic ETEC, in which inhabitants are chronically colonized with STa-producing organisms, have lower rates of CRC than geographic regions free of ETEC infections^[16]. These observations, coupled with the observation that guanylin and uroguanylin are universally lost along the CRC pathophysiological continuum, strongly suggest a role for GUCY2C as a tumor suppressor^[33,43,53].

Inflammation

Chronic intestinal inflammation, such as in IBD, is a well-known risk factor for CRC^[32,49,50]. *Gucy2c*^{-/-} mice demonstrate increased susceptibility to dextran sodium sulfate (DSS)-induced colitis^[21], 2,4,6-trinitrobenzene sulfonic acid-induced colitis^[52], as well as lipopoly-saccharide-induced intestinal injury^[54], while transgenic overexpression of guanylin (*Guca2a*+) renders mice resistant to DSS-induced colitis^[21]. Mechanistically, this effect appears to be mediated at least in part by maintaining intestinal epithelial integrity and barrier function^[21,48,49,54,55], since *Gucy2c*^{-/-} mice have reduced expression of proteins associated with tight-junction integral proteins, such as occludin, claudin 2, claudin 4, and JAM-A^[21,54]. Further, humans with IBD demonstrate significant decreases in guanylin, uroguanylin, and GUCY2C mRNA^[51,52]. Additionally, loss of these proteins has been linked with increased severity of IBD in patients^[51]. Together, these data suggest that GUCY2C signaling protects against chronic inflammation, and may play a critical role in inflammation-induced CRC.

Genomic stability and tumorigenesis

In addition to its anti-inflammatory effect, GUCY2C also helps maintain genomic integrity in the intestine, in both genetic (*Apc*^{Min/+}) as well as carcinogen [azoxymethane (AOM)]-induced mouse models of CRC^[18]. In both models, *Gucy2c*^{-/-} mice exhibited higher rates of tumorigenesis compared to mice with intact GUCY2C signaling^[18]. In addition, impaired GUCY2C signaling resulted in increased aberrant crypt foci, the earliest

precursors to CRC, when treated with the mutagen MNU compared to *Gucy2c*^{+/+} mice^[17]. Because both of these models rely on DNA damage to induce tumor formation, these findings suggest that GUCY2C signaling improves genomic stability. Indeed this was the case, as *Gucy2c*^{-/-} mice displayed increased DNA damage, measured by γ -H2AX, as well as an 8-fold increase in tumors with loss of heterozygosity of the *Apc* gene in *Apc*^{Min} mice^[18]. Taken together, these data demonstrate disruption of GUCY2C signaling is associated with genomic instability in the intestine that potentiates tumor initiation and growth.

Proliferation

The regenerating epithelium requires a reservoir of cells undergoing continuous cycles of proliferation in order to preserve intestinal functions^[56], while tight control of this proliferation is required in order to oppose tumorigenesis. Loss of GUCY2C function leads to dysregulated cell cycle progression^[17,18], as well as expansion of the proliferative compartment^[20]. Further, *Gucy2c*^{-/-} mice have upregulated expression or activation of oncogenes that promote proliferation, such as cyclin D1, pRb, β -catenin, and phosphorylated AKT (pAKT), as well as a decreased expression of tumor suppressors such as p27 and p21^[17,20]. In CRC cells, silencing GUCY2C promotes pAKT-mediated secretion of tumor growth factor beta (TGF- β), resulting in increased hepatocyte growth factor expression by submucosal fibroblasts, which feeds back to produce epithelial cMET signaling, an important driver of CRC proliferation^[57].

Reprogrammed Metabolism

Loss of GUCY2C signaling is also associated with metabolic reprogramming to a more glycolytic phenotype, an effect seen in many tumor types. For example, silencing GUCY2C increases expression of proteins associated with glucose transport (glucose transporter 1), glycolysis (hexokinase II), fatty acid synthesis (acetylCoA carboxylase) and lactate production, all of which are characteristic of the Warburg metabolic phenotype^[20]. Mechanistically, these effects appear to be mediated by GUCY2C-mediated inhibition of AKT, as constitutively active AKT eliminated the effects of GUCY2C activation and promoted a metabolic phenotype synonymous with the Warburg effect^[20].

The enhanced cell cycle progression, metabolic reprogramming and increased oncogenic signaling in *Gucy2c*^{-/-} mice also exists in human colon tumors^[12]. Importantly, loss of guanylin and uroguanylin occurs early in the progression of CRC, as would be expected in tumors mediated by impaired GUCY2C signaling. In fact, guanylin loss frequently accompanies APC loss in pre-malignant adenomas^[58]. While guanylin and uroguanylin expression is lost by nearly all colorectal tumors, the precise mechanisms mediating that loss,

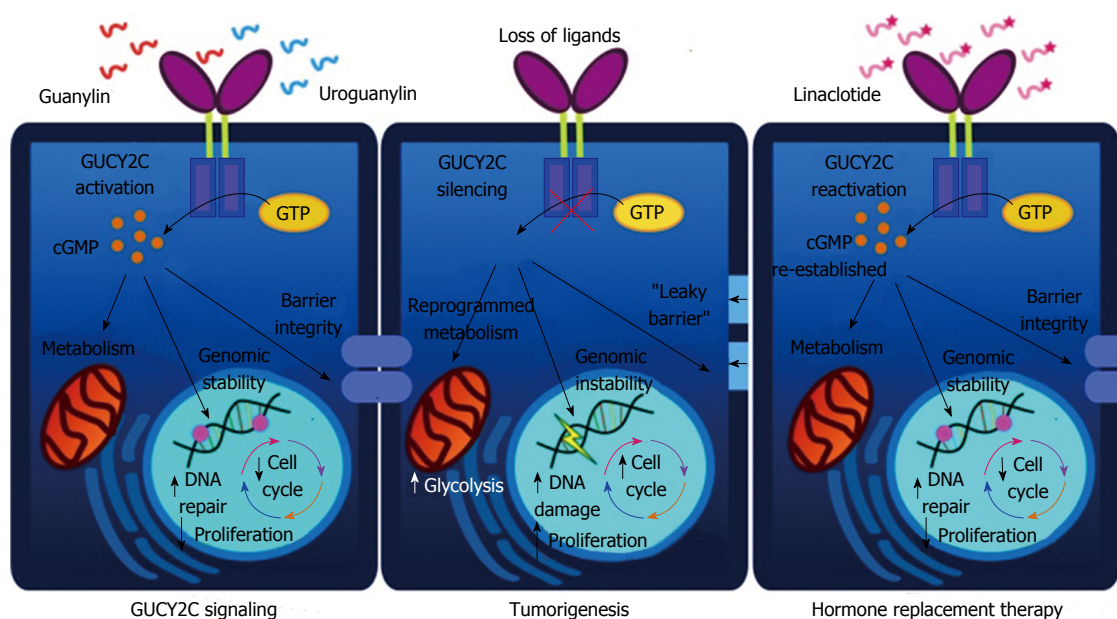


Figure 1 Guanylyl cyclase C receptor signaling, silencing, and reactivation. Ligand-mediated (guanylin and uroguanylin) activation of GUCY2C in the intestinal epithelial cell leads to accumulation of cGMP. This results in the maintenance of homeostatic mechanism, including regulated proliferation, barrier integrity, and proper cellular metabolism. Silencing of GUCY2C signaling by loss of hormone ligands leads to changes including increased glycolysis, increased proliferation, and a leaky intestinal barrier, all associated with colorectal tumorigenesis. These tumorigenic pathways potentially can be regulated with hormone replacement by oral supplementation with the analog linacotide which restores GUCY2C signaling to re-establish homeostasis. GUCY2C: Guanylyl cyclase C; cGMP: Cyclic guanosine monophosphate.

and the association of those mechanisms with the different etiologies of CRC (*e.g.*, APC mutation, MSI, CIN) remain to be defined. Nevertheless, accumulated evidence suggests a paradigm whereby loss of GUCY2C signaling represents a pivotal step in the progression of most colorectal tumors which, in turn, provides a potential therapeutic target for cancer prevention and/or treatment by GUCY2C hormone ligand replacement therapy.

TARGETED GUCY2C HORMONE THERAPY

Hormone deficiencies underlie a number of debilitating human diseases, including diabetes, thyroid disorders, and more recently CRC^[13]: Guanylin is significantly decreased (100- to 1000 fold) in nearly 90% of all CRC tumors colorectal tumors when compared to adjacent normal mucosa^[43]. The observation of a loss of guanylin expression in human colorectal tumors coupled with the potentiation of tumorigenesis observed in GUCY2C knockout mice supports a hypothesis suggesting that CRC initiates as a disease of hormone insufficiency and offers the possibility of a new paradigm for CRC treatment^[13,44,59]. Importantly, although loss of the GUCY2C ligands guanylin and uroguanylin occur early in tumor development, GUCY2C receptor expression persists in CRC^[60-62]. This suggests that the receptor is latent and that signaling can be reconstituted by ligand supplementation similar to other diseases of hormone deficiency in which reactivation of signaling using

synthetic ligand analogs have been therapeutic (Figure 1).

The feasibility of this prevention paradigm is underscored by the development and FDA-approval of linacotide (LiznessTM). Leveraging GUCY2C's established secretory function, linacotide was developed as an oral therapeutic for patients with constipation-type irritable bowel syndrome^[32,63]. Linacotide is a synthetic 14-amino acid peptide that shares structural homology with the guanylin peptide family^[64]. Linacotide's mechanism of action mimics the canonical GUCY2C signaling pathway by binding and activating the mucosal receptor to produce cGMP, supporting its utilization as a clinical therapeutic to re-establish the GUCY2C signaling axis that is lost in CRC^[65]. The use of linacotide to prevent CRC also has been implicated in a number of studies examining the protective functions of GUCY2C signaling. In human CRC cell lines and mice, treatment with ST and cGMP derivatives reduced the rate of cell division by increasing endogenous cell cycle inhibitors and decreasing cell cycle drivers previously discussed^[17-21,35]. ST treatment also improved the intestinal hyper-permeability found in *Gucy2c*^{-/-} mice, a phenotype associated with loss of barrier function and susceptibility to intestinal injury, by reconstituting the expression of several tight junction integral proteins^[21,54]. Moreover, oral uroguanylin reduced polyp formation in *Apc*^{Min/+} mice^[66]. Interestingly, a more recent study investigating the molecular mechanisms associated with diet-induced obesity observed that wild-type mice on a high-fat or high-calorie diet had increased levels of tumorigenesis to the same extent observed in *Gucy2c*^{-/-} mice^[19]. These mice exhibited

diet-induced guanylin loss that recapitulated the silencing of GUCY2C found in colorectal tumors as these mice showed elevated p-AKT, cyclin D1, and β -catenin^[19]. This study used a mouse model of intestinal transgenic guanylin expression (*VilGuca2a*⁺) to significantly reduce colon tumorigenesis induced by high-fat diet and AOM^[19], providing a genetic proof of concept for guanylin replacement in the prevention of CRC. In this context, oral administration of GUCY2C ligands has the capacity to prevent the molecular pathology associated with CRC, thus allowing for its translatability into the clinic.

CONCLUSION

CRC is widely accepted as disease of acquired sequential mutations leading to genomic instability and loss of epithelial integrity. However, the initiating events of epithelial transformation that give rise to the driving genetic mutations of CRC remain elusive. The GUCY2C signaling axis is thought to contribute to tumor initiation as its universal silencing by loss of its hormone ligands results in increased DNA damage, increased cell cycle progression, AKT activation, and deregulation of metabolic processes, which invariably potentiates tumor growth. The loss of GUCY2C hormones highlights CRC as a potential disease of paracrine hormone-insufficiency. Direct targeting of the GUCY2C pathway by hormone replacement therapy might restore homeostatic mechanisms required for normal intestinal function and potentially prevent CRC. The feasibility of hormone replacement therapy in the prevention and treatment of CRC is underscored by the development of the FDA-approved GUCY2C ligand linaclotide (LinzessTM), which can be orally administered to patients to restore GUCY2C activation. Other GUCY2C-specific peptide compounds currently are being developed, which will broaden the horizons of hormone replacement therapy for a number of gastrointestinal diseases, and could impact the way CRC is treated in the future. Thus, the paracrine-hormone hypothesis proposes a shift in paradigm for CRC development from irreversible genetic mutations to reversible signaling mechanisms that can be managed through oral hormone replacement therapy.

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Pediatric non-alcoholic fatty liver disease: Recent solutions, unresolved issues, and future research directions

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Abstract

Non-alcoholic fatty liver disease (NAFLD) in children is becoming a major health concern. A "multiple-hit" pathogenetic model has been suggested to explain the progressive liver damage that occurs among children with NAFLD. In addition to the accumulation of fat in the liver, insulin resistance (IR) and oxidative stress due to genetic/epigenetic background, unfavorable lifestyles, gut microbiota and gut-liver axis dysfunction, and perturbations of trace element homeostasis have been shown to be critical for disease progression and the development of more severe inflammatory and fibrotic stages [non-alcoholic steatohepatitis (NASH)]. Simple clinical and laboratory parameters, such as age, history, anthropometrical data (BMI and waist circumference percentiles), blood pressure, surrogate clinical markers of IR (acanthosis nigricans), abdominal ultrasounds, and serum transaminases, lipids and glucose/insulin profiles, allow a clinician to identify children with obesity and obesity-related conditions, including NAFLD and cardiovascular and metabolic risks. A liver biopsy (the "imperfect" gold standard) is required for a definitive NAFLD/NASH diagnosis, particularly to exclude other treatable conditions or when advanced liver disease is expected on clinical and laboratory grounds and preferably prior to any controlled trial of pharmacological/surgical treatments. However, a biopsy clearly cannot represent a screening procedure. Advancements in diagnostic serum and imaging tools, especially for the non-invasive differentiation between NAFLD and NASH, have shown promising results, *e.g.*, magnetic resonance elastography. Weight loss and physical activity should be the first option of intervention.

Effective pharmacological treatments are still under development; however, drugs targeting IR, oxidative stress, proinflammatory pathways, dyslipidemia, gut microbiota and gut liver axis dysfunction are an option for patients who are unable to comply with the recommended lifestyle changes. When morbid obesity prevails, bariatric surgery should be considered.

Key words: Non-alcoholic fatty liver disease; Childhood obesity; Non-alcoholic steatohepatitis; Hepatic metabolic syndrome; Non-alcoholic fatty liver disease diagnosis

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Core tip: Due to the high prevalence of obesity worldwide, non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of metabolic syndrome, represents the most common chronic liver disease in children living in industrialized countries. The present review summarizes the currently available knowledge on NAFLD pathogenesis, diagnosis and treatment, highlights new research achievements that will likely influence therapeutic strategies and discusses possible future directions in pediatric research.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease and liver-related morbidity and mortality worldwide. NAFLD has an estimated prevalence of 20%-30% in Western Countries and 5%-18% in Asia and is associated with the pandemics of obesity and excessive fructose consumption. Remarkably, the overall prevalence of NAFLD in children has reached approximately 10%, including up to 17% in teenagers and 40%-70% among obese children. While often benign and self-limiting, steatosis can progress with hepatocyte injury into non-alcoholic steatohepatitis (NASH) in 3%-5% of patients. NASH is characterized by lobular and/or portal inflammation, varying degrees of fibrosis, hepatocyte death and pathological angiogenesis^[1]. Although NAFLD progression to advanced fibrosis and cirrhosis is mainly a chronic phenomenon, liver transplantation has been described in youths^[2].

NAFLD PATHOGENESIS: OLD AND NEW FACTS

The "multiple-hit" hypothesis largely explains NAFLD

pathogenesis and progression^[3]. At disease onset, NAFLD is characterized by fat accumulation in the liver (steatosis) and insulin resistance (IR), which is heavily influenced by a sedentary lifestyle, hypercaloric diets, genetic susceptibility, and epigenetics; however, the interactions between these pathomechanisms remains poorly understood^[4,5]. Other associated comorbidities can develop in addition to liver damage. NAFLD is the hepatic manifestation of metabolic syndrome (MetS), which has been linked with obesity, IR, hypertension and hyperlipidemia.

Our review presents recent insights into NAFLD pathogenesis, diagnosis and treatment, underlines a number of new research achievements that are likely to impact therapeutic strategies and discusses possible future perspectives in pediatric research.

FAT ACCUMULATION IN THE LIVER AND ADIPOSE TISSUE

The liver does not normally store triglycerides (TG), but TG accumulation in the cytoplasm of liver cells is not necessarily toxic *per se*. Overeating causes a breakdown of physiological mechanisms that regulate energy harvesting in liver and adipose tissues (AT). In conditions of excessive feeding, an overload in hepatic fat accumulation exacerbates IR by interfering with the phosphorylation of insulin receptor substrates. Specifically, a key aspect of fat metabolism imbalance is the dysregulation of insulin signaling pathways, *e.g.*, sterol regulatory element binding protein 1; fatty acid translocase cluster differentiation protein 36 (FAT/CD36); and hormone-sensitive lipase, which leads to TG imbalance, fatty acid mitochondrial oxidation, and lipoprotein excretion and transport^[6].

Recent insights into lipotoxicity showed that fatty hepatocytes release large quantities of extracellular vesicles that transport bioactive molecules, including mRNA, non-coding RNAs, proteins, DNA and lipids, to target cells. The signals shuttled by extracellular vesicles control inflammation and fibrogenesis by recruiting or activating macrophages and quiescent hepatic stellate cells (HSCs), respectively^[1].

AT has emerged as an active and crucial player in the development of hepatic steatosis and chronic low-grade inflammation, and it constitutes a key link between obesity and metabolic dysregulation. TG-rich chylomicrons are mainly transported to peripheral tissues (80%), where free fatty acids (FFAs) are released and available for uptake *via* lipoprotein lipase (LPL). One of the strongest inhibitors of LPL is apolipoprotein C-III (ApoC-III)^[6]. In individuals with IR, insulin fails to efficiently suppress ApoC-III in the liver, which inhibits LPL action in peripheral tissues and favors the hepatic uptake of TGs-rich chylomicrons remnants.

In normal conditions, AT protects the body from excessive exposure to fatty acids. However, exhausting adipocyte expandability can produce lipotoxicity,

oxidative stress, and peripheral IR. Consequently, AT acquires a proinflammatory profile characterized by adiponectin suppression and the promotion of other adipocytokines, *e.g.*, adiponectin, leptin, resistin, and tumor necrosis factor- α (TNF- α), which leads to progressive liver damage. In particular, leptin can activate HSCs and suppress their apoptosis. Furthermore, peripheral IR independently predicts hepatic histological characteristics and alters the cardio metabolic risks in non-diabetic biopsy-proven NAFLD patients^[7].

OXIDATIVE STRESS

Excessive FFA influx to the liver, a known cause of hepatic IR, overwhelms the mitochondria and causes the accumulation of incompletely oxidized substrates, such as fatty acids, ceramide and diacylglycerides. Increased beta-oxidation reduces the availability of oxidized cofactors (NAD and FAD) and decreases the outflow from the respiratory chain, which leads to the accumulation of electrons, ROS production, and cellular damage. Mitochondria become the targets of ROS-induced damage. Oxidative changes in respiratory complexes impair their catalytic functions and cause mutagenesis of the mitochondrial DNA. This further worsens oxidative damage, leading to hepatocellular death and NASH progression^[8]. In addition, mitochondrial proliferation and differentiation, mostly regulated by the transcription coactivator peroxisome proliferator-activated receptor (PPAR)- γ -coactivator-1 α , can be impaired in NASH.

The oxidative stress induced by fatty acid overload in hepatocytes is derived from mitochondria, peroxisomes and microsomes. IR significantly increases peroxisomal oxidation because insulin is the principal inhibitor of cytochrome P450 4A (CYP4A), a key enzyme in this pathway. This inhibition amplifies cytotoxic ROS and lipid peroxidation. These products can diffuse into the extracellular space, influence Kupffer cells and HSCs and induce the nuclear-factor κ B (NF κ B) pathway, which causes the subsequent synthesis of TNF- α and several other proinflammatory and fibrogenic cytokines^[6].

SUGARS AND FAT ACCUMULATION

Over the last few decades, the progressively increased intake of added sweeteners has been associated with obesity, hypertension, and other components of MetS, including steatosis and severe liver damage. Obese children with NAFLD consume more carbohydrates when compared with their obese counterparts without NAFLD. High blood glucose levels play a major role in liver fat accumulation by activating carbohydrate response element-binding protein (ChREBP), which physiologically regulates glycolysis and *de novo* lipogenesis independently from insulin. Saturated fat stimulates fatty acid oxidation *via* a PPAR α -dependent

mechanism and *de novo* lipogenesis in the liver; however, the prominence of these effects depends on the sucrose content in the diet^[6]. Hypercaloric diets enriched in fat and fructose/sucrose may act either by favoring the occurrence of systemic IR and, in turn, a dangerous hepatic FA accumulation or by causing visceral fat deposition and abdominal obesity, which are independent risk factors for MetS^[9].

Fructose, a highly lipogenic sugar, is found in fruits and vegetables with a high fructan content (*e.g.*, artichokes, wheat, leeks, garlic) and honey. Fructose, sucrose and high-fructose corn syrup are used as added sweeteners, and the high consumption of these compounds has sparked increasing concern. Fructose metabolism differs from that of glucose because it is metabolized almost completely in the liver *via* GLUT5 and not by the insulin-dependent transporters GLUT1 and GLUT4; thus, fructose metabolism is relatively unregulated by insulin. Once in hepatocytes, fructose is mainly converted by fructokinase to fructose 6-phosphate, which is then hydrolyzed into fructose 1-6 biphosphate by fructose aldolase in order to enter glycolytic/gluconeogenesis pathways. Fructose metabolism induces a hepatic depletion of ATP with a consequent increase in AMP and uric acid. Although fructose does not acutely increase insulin, fructose can ultimately increase IR and fasting glucose and insulin levels. However, the mechanism of action remains to be determined^[10].

The metabolic destiny of ingested fructose is prevalently towards oxidation and conversion to glucose rather than to TG. Nonetheless, postprandial lipedema is higher after fructose consumption. Fructose determines the insulin-independent induction of several hepatic lipogenic enzymes (*e.g.*, pyruvate kinase, NADP⁺-dependent malate dehydrogenase, citrate lyase, acetyl CoA carboxylase, fatty acid synthase, and pyruvate dehydrogenase) and an increase in VLDL production and hepatic fat storage^[11]. High fructose intake is linked with hepatocyte apoptosis, hepatic fibrosis and dyslipidemia^[10].

In NAFLD patients, ingested fructose may alter the microbiome, which increases the movement of endotoxins into the portal system because of increased tight junction permeability. These changes increase liver inflammation and IR *via* Toll-like receptor (TLR) 4 activation. Moreover, obese children with NAFLD showed an increased absorption and exaggerated metabolic response to fructose administration when compared with lean controls. This effect may be due to the altered intestinal bacterial flora fermentation of fructose into hydrogen or an up-regulation of fructose transporter GLUT5 in the intestinal epithelium^[12].

GUT-LIVER AXIS AND GUT MICROBIOTA

The intestinal ecosystem has numerous physiological and pathological interactions with the host, including regulation of mucosal/systemic immunity and metabolic

and trophic functions. Intestinal microorganisms produce highly conserved molecules, such as pathogen associated molecular patterns (PAMPs) (*i.e.*, lipopolysaccharides/endotoxins), which are recognized by specific pattern recognition receptors (PRRs), including TLRs and NOD-like receptors. In NAFLD, an alteration in gut microbiota and enhanced gut permeability increase the exposure of the liver to PAMPs and/or other products of intestinal tissue injury, such as damage associated molecular patterns. Therefore, diet- and/or gut-microbiota-dependent increases in gut-derived products can cross the impaired gut permeability barrier, activate molecular mechanisms of innate immune response, and act as possible inductors of NAFLD progression^[13,14].

Conflicting qualitative/quantitative differences in bacterial flora composition have been found in obese subjects, possibly due to differences in methodology and/or host related factors (*e.g.*, immune system response and diabetes)^[15]. In obese adults, a functional study showed that the microbiota break down otherwise non-absorbable polysaccharides into monosaccharides and short chain fatty acids (SCFAs), which may provide extra calories. Monosaccharides activate the hepatic carbohydrate response element binding protein (ChREBP), which increases hepatic lipogenesis and fat accumulation; furthermore, SCFAs induce leptin production^[13].

Recent research suggests a critical role of the Farnesoid X receptor (FXR) in carbohydrate and lipid metabolism, regulation of insulin sensitivity and NAFLD pathogenesis. FXR is a nuclear bile acid (BA) receptor highly expressed in tissues that participate in BA metabolism, such as the liver, intestine, and kidneys. Gut microbiota can change the BA composition of the host, particularly taurine-conjugated BA, which can antagonize intestinal FXR and lead to metabolic dysfunction, including obesity and IR. BA can also influence NAFLD *via* activation of hepatic FXR and G protein-coupled receptor TGR5^[16,17].

ENDOGENOUS ALCOHOL PRODUCTION

The gut microbiota of healthy subjects produces trace amounts of endogenous ethanol (EE) from unabsorbed dietary sugars. In the liver, EE generates acetaldehyde, which in turn is oxidized to non-toxic acetate. In small intestinal bacteria overgrowth-related conditions, EE concentrations are significantly higher than in control subjects^[18]. Furthermore, recent pediatric studies suggest that EE in NAFLD patients correlates with increased intestinal permeability^[14]. Increased blood ethanol levels in patients with NAFLD might also result from an insulin-dependent impairment of alcohol dehydrogenase activity in liver tissue rather than an increase in EE synthesis^[19].

NAFLD-RELATED GENES

Mutations in several genes involved in lipid and glucose

metabolism, redox cellular state and inflammation can lead to hepatic steatosis. A single nucleotide polymorphism (SNP) of the patatin-like phospholipase 3 gene (*PNPLA3*), which encodes the insulin-regulated phospholipase adiponutrin, has been shown to be associated with both hepatic steatosis and inflammatory changes/fibrosis. Recent studies in pediatric cohorts showed that patients with the genetic variant *PNPLA3 I48M* (rs738409; C→G) are more likely to have NAFLD but not IR^[20]. Furthermore, a high sugar diet strongly interacts with the *PNPLA3GG* homozygous variant and predicts increased hepatic fat^[21].

A second gene particularly related to the progression of NAFLD to NASH is the G-protein-coupled-receptor 120 (*GPR120*), a receptor for polyunsaturated fatty acids (PUFAs) expressed by adipocytes, Kupffer cells and hepatocytes. The interaction between PUFAs and *GPR120* expression in macrophages reduces inflammation by inhibiting NF-κB activity. The *GPR120 270H* allele reduces the anti-inflammatory action of the GPR120 receptor. Therefore, *270H* carriers present pathological ALT levels due to liver injury caused by oxidative stress, mitochondrial dysfunction and overproduction of proinflammatory cytokines^[22]. Alimentary supplementation of docosahexaenoic acid (DHA), an n-3 PUFA in fish oil, has been recently suggested as a potential therapeutic. DHA activates the GPR120 receptor, which exerts potent anti-inflammatory and insulin-sensitizing activities^[23].

Under conditions of increased hepatic fatty acid influx or decreased efflux, PPARα activation prevents the accumulation of TGs by increasing the rate of fatty acid catabolism. The down regulation of the PPARα gene induced by the *Val227Ala* SNP was suggested to influence NAFLD pathogenesis; however, this effect was not histologically confirmed in an adult Italian population^[24].

PPARγ, a molecular target of glitazones, is highly expressed in AT and regulates adipocyte differentiation and FA uptake and storage. Controversial evidence has suggested a role for the *Pro12Ala* loss-of-function SNP in PPARγ in IR and liver disease progression^[24].

OTHER MECHANISMS INVOLVED IN NAFLD PATHOGENESIS

Iron and copper in pediatric obesity and NAFLD

Trace elements are critical in regulatory, immunologic, and antioxidant functions by protecting against inflammation and peroxidation. Disruption of the metal detoxification processes located in the liver are plausibly related to NAFLD development *via* oxidative stress. Perturbations of iron and copper (Cu) homeostasis have been shown to contribute to NAFLD pathogenesis^[25].

Ghrelin-ghrelin O-acyltransferase system in the pathogenesis of NAFLD

The ghrelin-ghrelin O-acyltransferase (GOAT) system

has been recently reported to play a crucial role in both the development of steatosis and its progression to NASH. The ghrelin-GOAT system is involved in IR, lipid metabolism dysfunction, and inflammation, all of which play important roles in the pathogenesis of NAFLD. The ghrelin-GOAT system is linked to energy and lipid metabolism, IR, inflammation, and apoptotic cell death, which are common to both obesity and NAFLD. Therefore, the role of the ghrelin-GOAT system in NAFLD has recently become a subject of considerable interest. The role of ghrelin in appetite regulation (orexigenic function) and energy metabolism is well established and is now recognized as a promising target for the treatment of obesity and NAFLD^[26].

Implication of vitamin D metabolism

Low levels of vitamin D are associated with obesity and NAFLD^[27]. Vitamin D receptors are expressed in a wide range of tissues, including the liver, and the immune system (*e.g.*, T and B cells, macrophages, and monocytes). Vitamin D has a paracrine/autocrine role in the regulation of cell proliferation, differentiation, apoptosis and immunity. A growing body of evidence suggests that low levels of 25(OH)D are strongly associated with MetS. These data are summarized in a recently published meta-analysis. NAFLD subjects were 26% more likely to be vitamin D deficient when compared with controls^[28]. It has been supposed that the progression of NAFLD caused by western diet is exacerbated by vitamin D deficiency. Specifically, activation of TLR2 and TLR4 *via* CD14/LBP and stimulation of downstream inflammatory signaling molecules leads to steatosis and inflammation. The metabolic, anti-inflammatory and antifibrotic properties of vitamin D provide plausible mechanisms by which vitamin D may influence disease progression and severity.

Obstructive sleep apnea syndrome

Obesity-related obstructive sleep apnea syndrome (OSAS) is considered to be a risk factor for more severe NAFLD. OSAS is a sleep disorder characterized by a complete or partial upper airway obstruction due to pharyngeal collapse during sleep, snoring, frequent nocturnal awakenings, sleep deprivation, and daytime sleepiness. Recently, several studies showed a correlation between OSAS and the progression of simple steatosis to steatohepatitis. This progression may be induced by a chronic intermittent hypoxia that promotes liver inflammation and fibrosis. Liver injury occurs through the enhancement of oxidative stress *via* the ischemia-reperfusion damage process and several molecular mechanisms, including the promotion of inflammatory cytokines (IL-1 and IL-6) in hepatocytes and macrophages by hypoxia-inducible factor and NF- κ B, which then modulates fibrogenesis and angiogenesis in Kupffer cells and HSCs^[29,30].

NAFLD DIAGNOSIS

The initial step of identification requires that primary care pediatricians calculate and record the BMI at every visit for every child that is potentially "at risk" for obesity and NAFLD. NAFLD is generally "a silent liver disease" because it can present without any warning signs, and only those who develop NASH with more severe liver damage will have some symptoms of chronic liver disease. In clinical practice, NAFLD is therefore usually suspected based on the findings of hypertransaminasemia and/or an ultrasonographic bright liver in an otherwise healthy child who is overweight (BMI between 85th and 94th percentile) or obese (BMI \geq 95th percentile). However, the full spectrum of histologic NAFLD can also be present with normal liver tests. Data from mainly adult studies^[31] show that NAFLD is frequently found in conjunction with other conditions, such as visceral obesity, hypertension, hyperinsulinemia, IR or diabetes, dyslipidemia with an atherogenic lipid profile, or fructose rich diet-related hyperuricemia. These conditions are also predictors of liver involvement and/or fibrotic progression. The available diagnostic procedures for NAFLD include a number of clinical signs, blood tests and imaging techniques^[32,33].

CLINICAL SIGNS

At clinical examination, the presence of acanthosis nigricans, increased waist circumference (WC) and hepatomegaly should be recorded as they may represent surrogate markers of IR (one of the pathogenic hits), central/visceral obesity and liver involvement, respectively. Furthermore, the combination of these features can suggest NAFLD risk.

Regarding WC, Lin *et al.*^[34] found that for every 5 cm increase in waist circumference there was an odds ratio of 1.4 for predicting ultrasonographic liver steatosis. Increased WC is also associated with increased hepatic fibrosis^[35,36].

Simple screening questionnaires or more specific polysomnography can be used to confirm/rule out OSAS, a novel pathogenetic factor of NAFLD in children^[37].

In female adolescents with NAFLD, a screening for polycystic ovary syndrome (PCOS) is recommended, especially when history and clinical examination show signs of hyper-androgynism (*i.e.*, acne, hirsutism, and irregular menstrual cycles). Pre-menopausal women with NAFLD present a high prevalence of PCOS, which may be because both diseases share common pathogenic mechanisms linked to MetS and obesity^[38].

Furthermore, the detection of NAFLD may be helpful for the early identification of individuals with an increased cardiovascular (CV) risk. In obese patients, liver involvement by itself may promote CV diseases independently of other MetS components.

Indeed, children with ultrasound-diagnosed NAFLD and hypertransaminasemia present functional and morphological vascular changes [*i.e.*, endothelial dysfunction, impaired flow-mediated dilation of the brachial artery, increased carotid intima-media thickness, and altered ventricular functions]. Patients with NASH are at higher risk for atherosclerosis due to peripheral IR, pro-atherogenic lipid profile, oxidative stress and systemic inflammation^[39]. Thus, blood pressure evaluation, control, and monitoring should be an integral component of the clinical management of children with NAFLD^[40].

SERUM LEVELS OF HEPATOBILIARY ENZYMES AND MOLECULES

Alanine aminotransferase: Although NAFLD is the most common cause of hypertransaminasemia in children and adolescents^[41], elevated Alanine aminotransferase (ALT) is not a sensitive marker of disease existence and/or severity at ordinarily used thresholds^[42]. According to the Screening ALT for Elevation in Today's Youth study, normal values of transaminases for teenagers and children are presently set too high to detect liver steatosis. The 95th percentile levels for ALT in healthy weight, metabolically normal, liver disease-free children should be 25.8 U/L for boys and 22.1 U/L for girls. With this cut-off, the diagnostic sensitivity raised from 32% to 80 % in boys and from 36% to 92% in girls^[43].

Aspartate aminotransferase: The evaluation of both Aspartate aminotransferase (AST) and ALT values is essential because an increased AST/ALT ratio can reflect a progressive and more severe condition, such as fibrotic NASH.

γ -glutamyl transpeptidase: High serum levels of γ -glutamyl transpeptidase (GGT) represent a risk factor for advanced fibrosis in NAFLD^[44].

Bile acids: Serum bile acids (BA) levels decrease in early NAFLD and increase during its progression to fibrosis. Given that BA levels are increased in cirrhotic adults, it has been postulated that the continuous rise in BA as NAFLD advances may have a value as a noninvasive biomarker for pediatric NAFLD progression^[45].

Glucose, insulin, the homeostatic model assessment-IR and lipid profiles: These should be evaluated in all children with suspected or diagnosed NAFLD.

IMAGING TECHNIQUES

Imaging methods, such as ultrasounds (US) and magnetic resonance imaging (MRI) \pm chemical shift imaging or spectroscopy, have been increasingly approved as noninvasive alternative methods to

diagnose and monitor NAFLD/NASH^[36,46]. (1) US is safe, but it is limited by the inability to detect fatty liver (liver brightness vs kidney parenchymal echogenicity) when steatosis involves < 30% of hepatocytes^[47]. US has several advantages for use as a screening tool: relatively low cost, large diffusion in medical community and feasibility. US has been used to assess the outcome efficacy in pediatric trials with good compliance in children and parents^[36,48]; (2) generally, MRIs are not cost-effective, even with certain modifications that could enable rapid and reproducible measurements of steatosis and fibrosis. With modern MR spectroscopy software, the measure of intracellular water and lipid content can be used to define hepatic steatosis if the hepatic TG/water ratio is > 0.5. Recently, advanced MRI for the quantitative assessment of hepatic steatosis was validated in a pediatric study that confirmed the correlation and diagnostic accuracy of MRI-estimated liver proton density fat fraction as a biomarker for hepatic steatosis when compared with histologic steatosis grade. Although magnitude-based MRI has the potential for clinical utility in the evaluation of NAFLD, there is no current single threshold value with sufficient accuracy for diagnosis of children^[49]; and (3) other radiologic imaging methods can estimate liver stiffness as a surrogate for liver fibrosis, including transient elastography (TE), magnetic resonance elastography (MRE), and acoustic radiation force impulse imaging (ARFI)^[50]. Perhaps the most promising imaging tool for differentiation between hepatic steatosis and NASH is MRE. A recent study underlined the utility of MRE for discriminating advanced fibrosis (stage 3-4) from mild fibrosis (stage 0-2) with excellent sensitivity (0.86) and specificity (0.91). In addition, magnetic resonance scanning allows an estimate of total fat tissue^[51].

LIVER BIOPSY HISTOLOGY

The histological spectrum of NAFLD ranges from simple steatosis to NASH and cirrhosis. Pediatric fatty liver disease often displays a pattern that is distinct from adults. Schwimmer *et al.*^[52] categorized NASH into three types according to the histological characteristics: adult type, pediatric type, and overlap type. The first was characterized by steatosis, ballooning degeneration, and perisinusoidal fibrosis. The second and third types (mainly found in children) were characterized by steatosis, portal inflammation, and portal fibrosis.

Liver histology remains the mainstay measure for clinical trials. Biopsies are used both for enrollment and determining the outcome of clinical trials. End points, such as reversion of NASH or regression of fibrosis, may require a clear histological definition. However, liver biopsies are not exempt from possible sampling errors, which can result in substantial misdiagnosis and staging inaccuracies because histological lesions of NASH may be unevenly distributed throughout the liver parenchyma. In 2012 the ESPGHAN panel^[36] indicated

Table 1 Non-invasive diagnostic tests of non-alcoholic fatty liver disease

Hepatic fibrosis scores	Advanced biochemical markers	Newly proposed markers
AST/ALT ratio	Cytokeratin 18 fragment levels (CK-18)	Serum potassium
Platelet ratio index (APRI)	Extracellular matrix turnover biomarkers:	Soluble Fas and Fas Ligand (sFasL)
Fibrosis (FIB)-4 index	Enhanced liver fibrosis (ELF) test	Plasma cathepsin D (CatD)
NAFLD Fibrosis score (NFS)	Amino-terminal propeptide III procollagen (PIIINP)	Circulating zonulin
Pediatric NAFLD fibrosis score	Hyaluronic acid (HA)	Adipokines (e.g., chemerin)
		Serum Uric Acid (UA)
		Vitamin D
		Proteomics signature
		Metabolomic signature

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NAFLD: Non-alcoholic fatty liver disease.

that although a liver biopsy is required for a definitive NAFLD diagnosis, its invasive nature and high cost prohibit the use of biopsies as a screening procedure. The same panel recently^[53] highlighted that indications for liver biopsy in NAFLD are still controversial and there is limited available evidence for the formulation of guidelines. Indications for liver biopsy are only based on expert opinions that must take into consideration a differential diagnosis and the risk of progression of liver disease to cirrhosis. For a differential diagnosis, a liver biopsy should be considered after noninvasive biochemical and metabolic tests have been completed.

The ESPGHAN panel generally accepted the criteria of Roberts *et al.*^[54] and summarized the indications for liver biopsy: to exclude other treatable disease, in cases of clinically suspected advanced liver disease, before pharmacological/surgical treatment, and as part of a structured intervention protocol or clinical research trial. In the most frequent age range of pediatric presentation (children older than 10 years), a liver biopsy should be considered if hypertransaminasemia or US hyperechogenicity persists after attempts at weight reduction and lifestyle changes for 3-6 mo and if the laboratory workup is still inconclusive. A liver biopsy should be completed even earlier in patients with a family history of NASH, hepatosplenomegaly, comorbidities, hypothalamic expansive processes, marked hypertransaminasemia, or elevated serum fibrosis markers.

NON-INVASIVE INDIRECT DIAGNOSIS OF NAFLD

A number of non-invasive diagnostic tests for NAFLD have been proposed and are summarized in Table 1.

Hepatic fibrosis scores: Noninvasive methods for diagnosing liver fibrosis in patients with NAFLD include simple fibrosis scores that use readily available laboratory tests (AST/ALT ratio, AST to platelet ratio index, fibrosis-4 index, and NAFLD fibrosis score). These scores were developed in adults and have poor performance in diagnosing advanced fibrosis in children with NAFLD. Recently, Alkhouiri *et al.*^[55] developed a

new "Pediatric NAFLD fibrosis score" (based on ALT, alkaline phosphatase, platelet counts and GGT) that appears more capable of predicting advanced fibrosis.

Advanced biochemical markers: hepatocyte cell death and extracellular matrix turnover:

Biomarkers of hepatocyte cell death, such as cytokeratin 18 (CK-18) fragment levels, can be used. Markers of extracellular matrix turnover, such as the enhanced liver fibrosis test that consists of tissue inhibitor of metalloproteinases 1, amino-terminal propeptide of type III procollagen and hyaluronic acid (HA), show good correlation with fibrosis stage^[56].

Newly proposed markers: Serum potassium levels show an inverse relationship with the presence of aggressive disease (NASH and fibrosis) in children with NAFLD^[57]. Markers of the extrinsic pathway of hepatocyte apoptosis [e.g., circulating soluble Fas and Fas ligand (sFasL) levels] that are elevated in children with NASH may be potential novel biomarkers^[58]. Plasma cathepsin D (CatD) holds a high diagnostic value for distinguishing pediatric patients with hepatic inflammation from children with simple steatosis. A reduction in CatD correlates better than ALT and CK-18 with pediatric disease progression, in terms of liver inflammation severity, steatosis grade, hepatocellular ballooning, NAFLD activity score, and this correlation reaches almost maximum accuracy after factoring in the CK-18 levels^[59]. Circulating zonulin is associated with the stage of liver disease in obese children with biopsy-confirmed NAFLD. Increased zonulin values correlated with steatosis severity^[60]. Serum levels of classic adipokines (e.g., leptin, adiponectin, and resistin) are altered in patients with NAFLD. Recently, the role of novel adipokines (e.g., chemerin, omentin, and vaspin) has emerged in NAFLD pathogenesis; however, their serum concentrations in obese children with NAFLD have been poorly studied. Chemerin appears to be the most suitable non-invasive biomarker for predicting intrahepatic lipid content^[61]. Serum uric acid (UA), as a surrogate marker for fructose intake, is significantly increased in children with NASH^[62]. Furthermore, elevated UA levels positively correlate with IR and the number of

Table 2 Fatty liver disease: selection of possible causes in children and adolescents^[41]

General or systemic	Genetic-metabolic causes	Drugs/chemicals
Anorexia nervosa (\pm refeeding)	α - and β -oxidation defects	Corticosteroids
Celiac disease	Abeta or hypobetalipoproteinemia	Diltiazem
Diabetes mellitus type 1	Alpha 1 -antitrypsin deficiency	Ecstasy, Cocaine, Solvents
Hepatitis C	Cholesterol ester storage disease/LAL	Estrogens
Hypothalamic-pituitary disorders	Citrin deficiency	Ethanol
Inflammatory bowel disease	Congenital disorders of glycosylation	Methotrexate
Obesity/Metabolic syndrome	Cystic fibrosis/Shwachman syndrome	Nifedipine
Obstructive sleep apnea	Familial hyperlipoproteinemias	Pesticides
Polycystic ovary syndrome	Glycogen storage disease (I , VI and IX)	Prednisolone
Protein calorie malnutrition	Hereditary Fructose Intolerance	Solvents
Rapid weight loss	Lipodystrophy	Valproate
Small intestine bacterial overgrowth	Mitochondrial and peroxisomal defects	Vitamin A
Thyroid disorders	Organic acidosis	Zidovudine and HIV treatments
	Porphyria cutanea tarda	
	Turner syndrome	
	Urea cycle disorders	
	Wilson's disease	

LAL: Lysosomal acid lipase.

MetS features^[63]. Recent data suggested the possible protective role of vitamin D against NAFLD and/or its progression in children. These findings underline the need to test for NASH as an early, obesity-related complication when serum vitamin D levels are persistently low in obese children. The serum level of vitamin D, even when within the normal range, has been found to inversely correlate with NAFLD severity, independently of known metabolic risk factors^[64]. Candidate proteomic biomarkers were identified in recent studies by mass spectrometry techniques. These studies, recently reviewed by Lădaru *et al*^[65], have identified 251 candidate proteomic biomarkers: thirty-three biomarkers were confirmed, 14 were found in liver samples, 21 in serum samples, and two from both serum and liver samples^[65]. A urinary metabolomic signature of pediatric obesity related liver disease, obtained by gas chromatography-mass spectrometry, showed distinct patterns that especially differed in the levels of metabolites involved in energy, peptide and organic acid metabolism, and intestinal bacteria metabolism^[66].

DIFFERENTIAL DIAGNOSIS

With the rising prevalence of childhood obesity, the proportion of children with both an underlying primary liver disease and NAFLD has also increased. Therefore, it is essential to identify conditions that are treatable with specific therapies, such as Wilson's disease^[67], autoimmune hepatitis^[68] or celiac disease^[69]. When pediatric NAFLD is suspected, other liver diseases should be excluded based on an age-driven algorithm^[36].

Abnormal serum aminotransferases in overweight or obese children are not always diagnostic of NAFLD/NASH, and other causes should be ruled out, including muscle diseases^[70] and treatable liver diseases^[67-69].

The ESPGHAN panel highlighted that hepatosple-

nomegaly is suggestive of an unusually advanced liver disease in pediatric NAFLD, and this condition requires a rapid and complete assessment, including an early liver biopsy, to exclude other etiologies^[36,54].

In general, obesity related-NAFLD does not occur in extremely young children (younger than 3 years of age) and is rare in children younger than 10 years of age. A differential diagnosis should be based first on clinical features and then on blood tests. As a final step, a liver biopsy must be considered. The remaining NASH-related conditions should also be carefully considered, especially in early-onset NAFLD among young children^[36,41]. Table 2 shows the main differential diagnoses in children and adolescents^[41].

TREATMENT

Lifestyle interventions (*i.e.*, diet and exercise) represent the mainstay treatment; however, compliance in both adults and children is poor. Research on the pathogenesis, genetic markers, and the role of gut microbiome in NAFLD has led to development of several medical and surgical therapeutic approaches. Figure 1 summarizes the currently available information.

Lifestyle changes

Lifestyle changes are the first line of intervention^[71,72]. This goal is not easy for most patients; thus, the recommended behavior is commonly unsuccessful. However, when successful, it represents the most safe and efficacious cure for overweight/obese children with NAFLD.

Diet and exercise act synergistically by improving both hepatic and extra-hepatic insulin sensitivity and restoring insulin metabolism pathways, especially those related to glucose and lipid homeostasis. Moreover, long-term lifestyle changes (24 mo) improve liver histology in terms of the grade of steatosis,

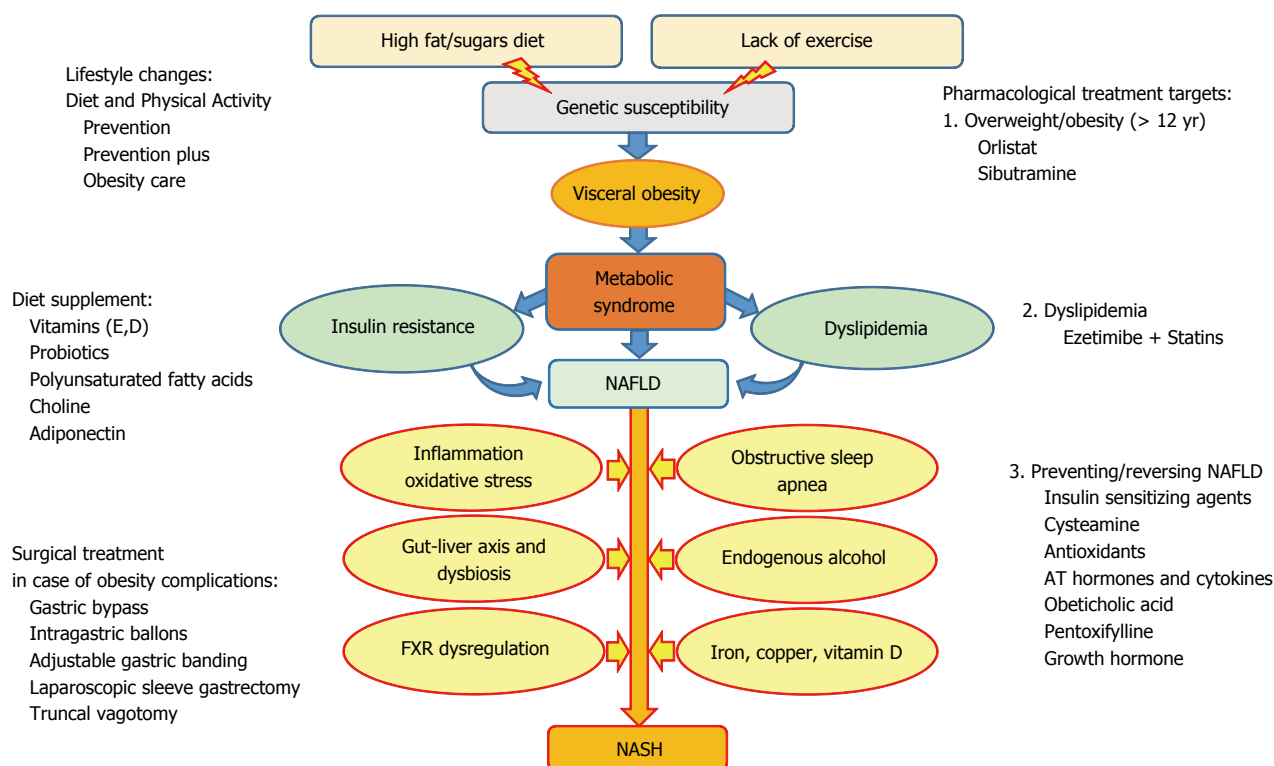


Figure 1 Simplified cartoon of the multiple pathomechanisms involved in development and progression of non-alcoholic fatty liver disease, and proposed stepwise treatment options. NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steato-hepatitis; FXR: Farnesoid X receptor; AT: Adipose tissue.

hepatic lobular inflammation, hepatocyte ballooning, and NAFLD activity score^[72].

Family involvement and effective interaction with the primary care pediatrician are critical for the success of lifestyle changes. In 2007, an expert committee nominated by the American Academy of Pediatrics (AAP) developed new recommendations for pediatric obesity prevention and treatment^[73]. The committee pointed out that awareness of the problem among primary care pediatricians who work with patients and parents is essential for the early identification of at-risk patients and for taking adequate preventive or corrective actions.

The expert committee highlighted the need for a radical change to the treatment of pediatric obesity, suggesting a "stepwise approach" that includes BMI assessments and the early identification of the risk factors for all children.

After identification and assessment, primary care pediatricians should proceed with the recommended measures of prevention or treatment, depending on the patient's age and BMI. The AAP expert committee identified three different plans of action: "prevention", "prevention plus" and "obesity care". These stages increase in intensity; thus, patients can begin with the less intensive plan and gradually move to the most intensive plan, depending on the response over an established period. "Prevention" applies to normal-weight children. It requires the yearly evaluation of high risk nutritional/activity behaviors and the

recommendations for both diet (e.g., 5 or more servings of fruits and vegetables per day and avoiding sugar-sweetened beverages) and physical activity (e.g., 2 or fewer hours of screen time per day, no television in the room where the child sleeps, and 1 h or more of daily physical activity).

"Prevention plus" applies to overweight children. The goal should be weight maintenance, which will reduce their BMI as the child's age increases. Recommendations for eating behavior include at least 5 to 6 family meals per week, allowing the child to self-regulate his or her meals and avoiding restrictive behaviors. The general rule to follow is "parents provide, child decides". Physical activity needs to be structured: 60 min of at least moderate physical activity per day and 20 min of vigorous activity 3 times a week. Referring families to community activity programs can be helpful and may encourage the development of family activities. Pedometer use can be recommended.

"Obesity care" (if BMI \geq 95th percentile) is a multidisciplinary team task involving several professional figures, such as a physician, nurse, dietician, exercise trainer, social worker and psychologist. In these cases, obese children must be referred to a pediatric tertiary weight management center with expertise in childhood obesity.

The goal is weight maintenance or gradual weight loss until their BMI is < 85th percentile and should not exceed 1 pound (= 453.6 g) per month. In cases

of severe obesity in older children (> 5 years) and adolescents (BMI \geq 99th percentile), weight loss may be more rapid with a maximum rate of 2 pounds (= 907.2 g) per week.

The expert committee identified in the following seven the behaviors that most successfully create energy balance: limit consumption of sugar sweetened beverages, limit TV (0 h for children < 2 years of age and < 2 h for children > 2 years-old), remove TV from primary sleeping area, eat breakfast daily, limit eating out, encourage family meals, and limit portion size^[73].

Micronutrient diet supplementation

The benefits from adding otherwise insufficient micronutrients to the diet, including vitamin E and D, probiotics, polyunsaturated fatty acids, choline, and adiponectin, appear to be short-term. The long-term effects of micronutrient supplementation are controversial and further studies are required.

Vitamin E was the first diet supplement used in pediatric NAFLD and NASH because of its anti-oxidant properties^[74,75]. Recent results from the "Treatment of NAFLD in Children" (TONIC) multicenter randomized placebo-controlled trial showed that vitamin E was not superior to the placebo at attaining the primary outcome of sustained reduction in ALT levels in patients with pediatric NAFLD^[76]. However, children treated with vitamin E demonstrated significant improvements in the resolution of NASH in patients with NASH or borderline NASH at baseline when compared with placebo. Therefore, vitamin E treatment in children who failed to change their lifestyle and have biopsy-proven NASH can be recommended^[76].

Diet supplementation with PUFAs, such as the omega-3 fatty acids DHA and eicosapentaenoic acid (EPA), restore insulin sensitivity and have anti-inflammatory actions^[77,78]. A six-month diet with long chain omega-3 fatty acids induced a significant decrease in AST and GGT, but not ALT^[79]. TG and ALT levels respond to DHA. The benefit of diet supplementation with PUFAs was confirmed in adolescents with NAFLD and genetic LPL deficiency, which has a high prevalence among the French-Canadian population in Quebec and is associated with a high risk of atherosclerosis early in life^[80].

Vitamin D deficiency has been suggested to promote NASH by accelerating hepatic fibrogenesis^[28]; thus, supplementation with vitamin D has been recommended^[81].

Healthy humans are unable to synthesize enough choline *de novo* to prevent deficiency. Decreased choline intake is associated with worse fibrosis in a subset of patients with NASH. Studies have shown a reversal of NASH after choline supplementation in choline-deficient patients on long-term parenteral nutrition. Additional studies are needed to evaluate if low choline concentrations are associated with the initiation or progression of NAFLD or NASH^[82].

Probiotics

The type of food ingested has an enormous influence on gut microbiota composition. Approximately 10 years ago, research studies began to demonstrate how gut microbiota are involved in significantly regulating energy balance, including reducing AT and cholesterol blood levels^[83]. Compared to slim subjects, the obese have different gut microbiota, which also varies depending on the geographical area. Changes in the gut microbiota using prebiotics (substances useful to the growth of good gut microbiota) and probiotics (live microorganisms) are beneficial for weight reduction^[83,84]. Extensive experiments conducted on animals showed strain-specific effects. In NAFLD, diet supplements studies included in particular the genera *Bifidobacteria* and *Lactobacillus*^[85,86]. *Lactobacillus rhamnosus* has been extensively studied in NAFLD both *in vitro* and *in vivo*. This strain modulated gut microbiota, reversed small intestinal barrier impairments, reduced hepatic inflammation, improved lipid metabolism and, probably most importantly, increased the production of certain anorexigenic gut hormones^[87,88]. The results of a recent double-blind clinical trial showed the benefits of the multi-strain probiotic VSL#3 on weight reduction and liver fibrosis in obese children with NAFLD aged 6-12 years^[89]. A double-blind clinical trial demonstrated that obese children with NAFLD treated with *Lactobacillus GG* showed a significant decrease (up to normalization in 80% of cases) in serum ALT values^[90]. A recent meta-analysis confirmed that experimental treatment with probiotics is more effective than placebo at normalizing transaminase levels^[91].

MEDICAL THERAPY

The pharmacological treatments currently available in pediatric NAFLD are mainly aimed at (1) reducing body weight; (2) preventing or reversing hepatic steatosis, inflammation and fibrosis; and (3) treating hypercholesterolemia.

Pharmacological treatment of the overweight/obese status

Candidates for pharmacotherapy are very high-risk children. These are children in the 99th percentile BMI at their first evaluation and children with a lower BMI who failed to change their lifestyle over a period of at least 6 mo. Tertiary care centers with specific expertise should take the care of children in these categories. The only two products currently approved by the Food and Drug Administration (FDA) for the treatment of pediatric obesity are orlistat and sibutramine^[73].

Orlistat: The human intestine does not absorb orlistat. The mechanism of action for this drug is based on the inhibition of endoluminal lipase. Orlistat was approved by the FDA for children above 12 years of age^[73]. Apart

from the common side effects of abdominal cramps and flatulence due to the amount of not absorbed fat in the fecal mass, orlistat can lead to chronic kidney disease due to secondary hyperoxaluria^[92]. Therefore, the use of orlistat must be carefully monitored, and patients must follow a low oxalate and high calcium diet with abundant daily water intake up to 1.5 L/m² body surface area^[92]. Probiotics able to eliminate oxalate and medications increasing the urinary solubility of crystals (e.g., potassium citrate), should also be considered.

Sibutramine: A nonspecific reuptake inhibitor for serotonin, norepinephrine and dopamine reduces appetite. Its use is approved for adolescents over 16 years of age for a period of no more than 2 years^[73]. The main side effect of vasoconstriction precludes its use in children with blood hypertension.

Medical therapy for preventing/reversing hepatic steatosis, inflammation and fibrosis

Treatments for NAFLD and NASH include insulin sensitizers, antioxidants and hepatoprotective agents. Their efficacy remains controversial in adults and in children as well^[93-95].

Metformin: It is an insulin sensitizer that could also improve weight control. The results from the TONIC study, a recent large multicenter randomized double-blind trial, showed no significant improvement of ALT levels or liver histology in NAFLD children aged 8-17 years on metformin vs placebo^[76].

Cysteamine: It's a coenzyme A catabolism product, has antioxidant properties and an insulin-sensitizing effect by up-regulating adiponectin levels. It reduced ALT and AST levels in children with NAFLD without reducing their body mass index. A randomized clinical trial is currently evaluating the potential effects of cysteamine bitartrate delayed-release capsules on the histologic severity of NAFLD in children aged 8-17 years; however, the results of this trial have not yet been published^[96].

Antioxidants: Vitamin E studies were previously mentioned in the "Micronutrient diet supplementation" section of this review. Among the plant extract polyphenols, resveratrol reduces hepatic inflammation and ameliorates lipid metabolism in adults^[97]. An ongoing clinical trial is evaluating the ability of resveratrol to reduce hepatic TG content in 13-18 year-old adolescents with NAFLD and metabolic syndrome^[98].

Silibinin: It's a drug available on most of the markets, is of particular relevance since its clinical potential has been extensively demonstrated in various experimental models^[99], and in a randomized clinical trial in adults with histologically documented NAFLD

where treatment was associated with improvement in liver enzymes, insulin resistance, and liver histology, without increases in body weight^[100].

AT hormones and cytokines: Adipocytes are now recognized to play an active role in MetS *via* the production of hormones, such as adiponectin and resistin, and adipocytokines, including TNF- α . These molecules are under investigation as possible therapeutic targets^[101].

Pentoxifylline: Pentoxifylline is a phosphodiesterase inhibitor that increases cyclic AMP and decreases TNF- α gene transcription^[102]. The results of a clinical trial showed that pentoxifylline is well tolerated and improves hepatic histology in adults with NASH^[103].

Obeticholic acid: The FLINT (FXR Ligand Obeticholic Acid in NASH Treatment) trial provided promising results on the efficacy of the obeticholic acid, an FXR agonist, at improving the histological features of hepatic inflammation and fibrosis in adults with NASH^[104].

Growth hormone: There is evidence that growth hormone (GH) replacement therapy improves serum liver enzyme levels and hepatic histology in adults that suffer from acquired GH deficiency and NASH^[105]. There is also a pediatric case report of an 11 year-old child suffering from acquired GH deficiency and NASH whose liver enzymes levels improved during GH treatment^[106].

Medical therapy for the treatment of hypercholesterolemia

Ezetimibe: Selectively inhibits cholesterol absorption from small intestine by binding to the brush border. The FDA has recently approved its use for treatment of hypercholesterolemia^[107]. Studies on *Ezetimibe* in NAFLD have shown improvement in hepatic histology but with worsening or no effects on insulin sensitivity and HbA1c levels^[108,109]. Recent studies on its safety and effectiveness in adult patients reported promising results when used in combination with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (*statins*)^[109,110].

SURGICAL THERAPIES FOR WEIGHT LOSS: BARIATRIC SURGERY

The number of surgical options has grown over the past decades, and these procedures are now used for weight loss in adults. Among the variety of techniques, there are strategies aimed to reduce gastric volume, food absorption and induce early satiety (gastric bypass, intragastric balloons, adjustable gastric banding, laparoscopic sleeve gastrectomy, etc.). Other strategies

create gastric stasis with an early sense of satiety, such as the truncal vagotomy^[111-113]. Even if bariatric surgery is still considered investigational in adults, it has been suggested for adolescents with moderate/severe obesity when complicated by diabetes, OSAS, pseudotumor cerebri or NASH or when an impaired quality of life and daily activities exists^[114,115].

FUTURE RESEARCH DIRECTIONS, PERSPECTIVES AND CONCLUSIONS

The epidemics of pediatric obesity and obesity-related liver disease (NAFLD and NASH) represents a serious problem. Increasing evidence indicates that affected children are at risk of significant progressive hepatopathy if inflammation and/or advanced fibrosis are already present.

The large diffusion of NAFLD does not consent a precise disease assessment of inflammation and fibrosis through liver biopsy in all individuals. Several non-invasive surrogate methods of diagnosis and surveillance are being developed with increasing sensitivity and specificity. Identification of NAFLD is also paramount for the early detection of other obesity related severe complications, *e.g.*, cardiovascular problems.

The existence of multiple pathogenic mechanisms may explain the resistance of NAFLD to standard treatments. A combination of several concomitant environmental and genetic factors, including the gut microbiota, leads to excessive accumulation of lipids in the liver (steatosis), which can often result in lipotoxicity, hepatocyte cell death, liver inflammation, fibrosis, and pathological angiogenesis.

Adult and pediatric NAFLD does not have a globally efficacious treatment. Lifestyle interventions (*i.e.*, dieting and exercise) represent the mainstay treatment, although effective interventions in adults and children are difficult due to a lack of compliance. Multiple medical treatments targeting IR, oxidative stress, gut liver axis and microbiota, proinflammatory pathways, and dyslipidemia can be proposed in patients that cannot adhere to the recommended lifestyle changes. Prevailing cases of morbid obesity may require bariatric surgery.

Knowledge on the pathogenesis, genetic markers, and the role of the gut microbiome in NAFLD continues to increase, which will likely lead to more effective treatment strategies. Obeticholic acid in adults appears to be a promising treatment; however, long term results are not yet available. In all cases, this type of therapy will require caution in adults and children due to the high expression of FXR in several tissues other than liver possibly showing beneficial and/or detrimental effects.

Last but not least, the prevention of obesity in both children and adults remains obviously imperative^[73,116,117].

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Epidemiology of hepatitis C in Greece

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Abstract

Hepatitis C is a global health issue and constitutes a major cause of chronic liver disease worldwide. In this article, a comprehensive literature search was conducted for the prevalence of hepatitis C virus (HCV) infection in Greece, since data on the HCV prevalence, viremia and genotypes are important for developing strategies to manage or eliminate HCV infection. In addition, the pattern of HCV infection was analyzed according to the geographic region and the risk factors. These differences reflect not only distinct epidemiological characteristics among populations, but also differences on the strategy of data acquisition and quantification. Although there are not enough data, the estimation of the current prevalence of Hepatitis C in Greece ranges from 0.5% to 2%. The most important risk factors for HCV infection include blood product transfusion, intravenous drug use, chronic hemodialysis, organ transplantation, occupational exposure, sexual transmission, and vertical transmission. Because of lack of vaccine or effective post-exposure prophylaxis for HCV, the main focus of prevention is to recognize and control these risk factors. HCV infection in Greece is closely associated with the development of chronic liver disease, cirrhosis, and primary hepatocellular carcinoma. As far as the genotype distribution is concerned genotype 1 estimated to be 45%-47% and it constitutes the prevalent genotype in Greece, followed by genotype 3.

Key words: Prevalence; Hepatitis C virus infection; Hepatitis C; Greece; Epidemiology

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Core tip: Hepatitis C virus (HCV) is a major health issue, it constitutes one of the most often causes of liver disease not only in Greece but also worldwide and is a potential cause of morbidity and mortality in the future. Epidemiological studies in Greece suggest that prevalence of HCV infection varies from 0.5% to 2%. The annual average incidence of HCV infection is estimated at 0.62 in 100000 people without significant difference between the two genders. These data are particularly important because they contribute to a better apprehension of the HCV infection in Greece since health authorities are able to create a more efficient health policy in a country which is hard affected from the economic crisis.

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INTRODUCTION

Hepatitis C is a contagious liver disease and constitutes a major global health issue. Hepatitis C virus (HCV) is a hepatotropic RNA virus which was discovered in 1989. It is a small, enveloped, single-stranded, positive-sense RNA virus and the structural domain contains two regions, the core and the envelope (E1 and E2). Approximately 60%-80% of infected people may progress to chronic liver disease and 20% of them will develop cirrhosis^[1]. Eventually, 5%-7% of these patients die from HCV-related complications^[2].

Parenteral exposure to HCV is the main cause of HCV transmission and it occurs *via* contaminated blood exposure, sexual transmission or vertical transmission from mother to child during perinatal period^[3]. Before 1990 the group with the highest risk of HCV transmission was the transfused patients. However, the post-transfusion HCV related hepatitis decreased dramatically, when the virus was isolated and the screening of blood and blood products carried out. Nowadays, in developed countries the most common route of HCV infection is through the use of drugs^[4]. Other groups of great risk are patients with history of surgical procedures (including abortion or uterine curettage), dental surgical procedures, chronic hemodialysis and tattooing^[5]. Sexual transmission and vertical transmission are less common ways of HCV infection^[3].

According to World Health Organization and recent studies, about 3% of the global population has been infected with HCV^[1,6,7]. It is estimated that chronic carriers of HCV are more than 170 million and they constitute a high risk group for developing cirrhosis and Hepatocellular carcinoma (HCC)^[7]. In addition,

at Eastern Mediterranean Region, 17 million people suffer already from chronic hepatitis C and 800000 individuals are infected each year with HCV^[8]. However, the epidemiological patterns among different countries of Eastern Mediterranean Region are not similar. Prevalence of HCV infection in countries such as Libya and Lebanon is reported to be 1.5%-3%^[8], whereas in other countries, such as Egypt, prevalence is much higher (14%-20%)^[9]. Epidemiological studies in Greece suggest that prevalence of HCV infection varies from 0.5% to 2%^[10-13]. Consequently, epidemiological data depend not only on the geographical region and the population group but also on the strategy of data acquisition (national registries, phone surveys, etc.) and quantification. This procedure points out the need for more robust epidemiology studies which will be able to quantify HCV in the general population taking into account the urban, rural and marginalized populations [Intravenous drug users (IVDUs), people in institutions, etc.].

We have conducted a literature review from January 1992 to January 2016 using Medline, Scopus, and Google Scholar databases and an amount of information was collected from the official site of Hellenic Center for Disease Control and Prevention (HCDCP).

PREVALENCE OF HEPATITIS C IN GENERAL POPULATION

The estimation of current prevalence of Hepatitis C in Greece ranges from 0.5% to 2%. HCDCP reports that from 1998 to 2011, 200000 individuals have been infected with HCV^[3] and 958 individuals have been infected with acute hepatitis C. The annual average incidence of HCV infection is estimated at 0.62 in 100000 people^[3] without significant difference between the two genders^[5]. A new epidemiological study which cites data from a recent phone survey^[14], reports an age-adjusted anti-HCV prevalence of 1.79% (1.87% high-risk individuals-adjusted) for the population of the study (ages 18-70), and a total estimated prevalence of 1.47%^[15]. According to the above studies, 58% of diagnosed chronic HCV patients have never been treated. This corresponds to approximately 15700 treated patients in 2011.

Furthermore, 16% of the total liver transplantations performed in Greece in the recent years (2011-2013) were attributable to HCV^[15]. Another study which included multinational study groups, reported an estimated 133000 total number of viremic infections in 2013 and it forecasted a decline to 106700 viremic infections in 2030^[16].

Prevalence of HCV infection depends on the geographical regions of Greece. For example, prevalence in Crete is higher than the average prevalence reported in mainland Greece^[5,17,18]. More specifically the highest prevalence of anti-HCV antibodies is reported in

Rethymnon (0.52%), whereas the lowest percentage of seropositivity is reported in Chania^[19] (0.23%). Furthermore, Thessaly, in central Greece, is a region with low prevalence of Hepatitis C (0.34%). However, there are three municipalities in Thessaly (Nea Ionia in Magnesia and Palamas and Sofades in Karditsa) where the percentage of anti-HCV antibodies is high^[20]. Moreover, some areas in North-Western Greece, such as Achaia (in Peloponnese) and Corfu are mentioned as regions with low prevalence of HCV infection (0.5%)^[12,21-23]. Another study which conducted in a Greek island called Zakynthos with a well-defined mixed (urban and rural) population, identified that the overall anti-HCV prevalence was 1.25%, while there was a significant higher prevalence (6.8%) in a well-defined rural area^[24]. A more recent study about the endemicity of viral hepatitis C in Cretan-population indicates that there is a higher prevalence of anti-HCV among younger individuals^[17]. It is worth mentioning that in the same area the hepatitis C markers are absent in the children. This fact may raise some questions about the possible sources of transmission of hepatitis C during the preceding years such as drug or alcohol abuse, tattoos or piercing^[25].

PREVALENCE OF HEPATITIS C IN HIGH RISK GROUPS

Transfused patients

In the 1970s and 1980s blood transfusion was the most common way of HCV transmission (known as Non-A, Non-B at the specific timeline)^[26]. Lauer *et al.*^[27] in 2001 noted that the probability of post-transfusion HCV transmission amounts to 1 case per 103000 blood units. However, the most recent screening methods for HCV in blood banks appreciate that the risk of transmission in the modern era is significantly decreased and it considered to be about 1 chance per 2 million units transfused [European Centre for Disease Prevention and Control (ECDC)]. No survey has been conducted about the risk of post-transfusion HCV transmission in the Greek population. Furthermore, among blood donors, the regular systematic donors have lower anti-HCV prevalence than the military and replacement (family) donors (percentage of 0.23%, 0.26% and 0.42% respectively)^[28].

One study which conducted in Greece revealed that from 434 patients infected with HCV, 167 (38.5%) had a history of blood transfusion^[4]. Nonetheless, study groups of the above trial included patients with post transfusion hepatitis (PTH) acquired prior to the 1990s (and thus before the era of systematic blood screening), a time point characterized by the expansion of IVDUs in Greece^[4]. In this study, it has been also reported that the genotype 1 was the most prevalent genotype in PTH patients with a prevalence of 57% prior to 1981, followed by a decrease to 48% post 1981 and a subsequent increase of genotype 3 from

13% to 30%^[4]. Another study, which also conceals the rapid epidemiologic expansion of genotype 3 in this time-frame in the general population, reports that the reduction of the prevalent genotype is due to the fact that individuals infected by transfusion are less likely to practice high-risk behaviors^[29].

There are other groups of patients, such as patients with β -thalassemia, who constitute an especially high risk group. In transfusion-dependent thalassemia, prevalence of HCV infection is very high. Various studies suggest that the percentage ranges from 23% to 74% depending on the number of patients at the studies^[30-32]. Nevertheless, chronic hepatitis C does not impair the overall survival of patients with transfusion-dependent thalassemia^[30].

Intravenous drug users

Considering that the blood screening has significantly reduced the possibility of disease transmission *via* transfusion, intravenous drug use is an important risk factor of HCV transmission^[9,33,34]. Many epidemiology studies in Greece suggest that 20%-40% of individuals with chronic hepatitis C have history of intravenous drug use^[4,9-11]. In addition, studies report that approximately 73% of IVDUs suffer from HCV infection as well as that 11% of the total HCV infections come from active IVDUs^[35]. The present socioeconomic status in Greece seems to affect negatively the prevalence of HCV among IVDUs. Data before and during the economic recession in Greece (2008-today)^[16] report that the decline in Gross Domestic Product was inversely correlated with the annual prevalence rates of human immunodeficiency virus (HIV) and HCV among IVDUs^[36]. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and the ECDC confirm this evidence^[37].

The exact rate of HCV-HBV co-infection in the IVDU group is not well known. A study reports that the prevalence of HbsAg positive patients constitutes the 5% of the whole IVDUs, while at the same time the prevalence of anti-HCV positive patients is about 73% of the mentioned group. The expected prevalence of co-infection in this group does not agree with the rather high rates reported in the international literature^[38].

HCV infected IVDUs are more frequently male, from urban areas as opposed to rural or semi-rural areas, with higher education, younger at study entry and less frequently immigrants^[11]. Particularly after 1992, nine out of ten HCV infections belong to the IVDUs group and one out of two IVDUs were younger than 20 years when they acquired the infection. Some proof of the aforementioned speculations can be sought in the multinational/multicenter study conducted by Hatzakis *et al.*^[16]. Experienced stuff determined the outcomes in each country. Data from Greece forecast an increase in the number of HCC cases to 35% by 2030. The number of liver related deaths will likewise be increased to 35%, while decompensated cirrhosis

and compensated cirrhosis infections will be increased 30% and 15% respectively^[16]. The results from this study concerns the general population but to some extent they reflect the younger age of infection's acquisition and thus the prolonged course of disease and the increase in the disease burden. In the present era international guidelines encourage physicians to treat HCV infected IVDUs since this strategy reduce the disease burden.

The prevalent genotype seems to be genotype 3 and especially subtype 3a, followed by genotypes 1a and 4a^[4,29,33,35]. Savvas *et al.*^[4] suggest that after 1981 prevalence of genotype 1 was reduced and prevalence of genotype 3 was increased. This change has been observed both for IVDUs and transfused patients.

The age of drug users and the duration of drug usage increase the possibility of HCV infection in IVDUs^[5]. However, the most HCV infections come from needle sharing among the drug users^[39]. A recent study suggests that during the period 2005-2006 the number of IVDUs did not change but needle sharing became more common^[11]. This phenomenon commonly occurs among prisoners who are IVDUs and for this reason they develop a higher rate of HCV infections in relation with the general population^[39,40]. As far as the prevention measures are concerned, it is evident that opioid substitution therapy and needle/syringe programs are among the most effective measures for preventing infectious diseases among IVDUs. Data from European monitoring services (EMCDDA and ECDC) in 2011 underline the fact that Greece is among the countries which use less the above prevention strategies^[37]. Specifically, Greece is placed 13th on the list of 18 European Union/European Economic Area (EU/EEA) countries on the topic of opioid substitution treatment, and 9th on the list of 12 EU/EEA countries.

Finally, high prevalence of anti-HCV antibodies (9%) is reported among patients in a Psychiatric hospital, and it is about ten times higher relative to general population. This is observed due to psychiatric morbidity since they have more possibilities to use intravenous drugs which are associated with HCV infection. In addition, the treatment for HCV is related to psychiatric adverse events^[41].

Patients in chronic hemodialysis

HCV infection is common among Greek patients with end-stage renal disease. Prevalence of anti-HCV ranges from 10% to 65% depending on the geographical region^[42]. Boletis *et al.*^[43] suggest that anti-HCV prevalence among hemodialysis patients of Laiko General Hospital of Athens is 24.3%. In a study of 366 hemodialysis patients, HCV infection was detected in 36%^[44]. HCV infection in hemodialysis patients occurs due to prolonged vascular access and exposure to infected patients and contaminated equipment. After

the implementation of universal precautions, the incidence of seroconversion was reduced to 0.56% and then to 0%^[42]. It is reported that prevalence of HCV transmission is related to hemodialysis duration and number of transfusions^[42,43,45]. In renal transplant patients, the prevalence of HCV infection is similar to hemodialysis patients^[43]. The most prevalent genotype concerning hemodialysis patients differs in various studies. According to Rigopoulou *et al.*^[44] and Diamantis *et al.*^[46] genotype 3a is the most common prevalent genotype. However, Griveas *et al.*^[42] suggest that the majority of HCV infections is genotype 1b. Katsoulidou *et al.*^[47] in an earlier study observed that genotype 4 is the most common genotype of HCV infections.

Health care personnel/Warship personnel

Health care workers and other groups such as policemen, prison staff and social workers constitute a high risk group for HCV exposure^[48]. The participation of the staff in invasive procedures such as surgeries, hemodialysis and venipunctures is a great importance risk factor^[26]. Each patient has 0.024% possibilities to infect another individual, whereas an HCV-infected patient has 1.8% possibilities to transmit the HCV virus^[48]. According to a Greek study, exposure to HCV can occur by percutaneous injury (82%), mucosal exposure (14%), excoriations and abraded skin exposure (3%), and by human bites (1%)^[48]. Prevalence of HCV infection among health care workers is estimated to be 1%^[26] and it seems to be lower in relation to the prevalence in general population^[48]. It is worth mentioning that many cases of occupational exposure are not reported. In addition, Falagas *et al.*^[49] demonstrate that the risk of a percutaneous exposure is bigger in health workers and nurses than doctors.

Mazokopakis *et al.*^[50] examined if there are a link between the seroprevalence of hepatitis A, B and C among the warship personnel in Greece. As far as the HCV prevalence is concerned, seems that there is no association between this group of individuals and HCV, since the anti-HCV prevalence in this group is similar to those reported in a Greek study among blood donors (0.5%-3%).

Diabetic patients

Allison *et al.*^[51] studied the association between diabetes mellitus (DM) and HCV and they noted that 50% of HCV positive patients developed DM, in contrast to 9% of HCV negative patients. Thus, they proposed that may exist a direct effect of HCV upon pancreatic islet cells or an autoimmune procedure. In a same study in Greece the results were opposite since they noted low anti-HCV prevalence in diabetic patients (1.65%). According to this study the diabetic population cannot establish as a group at high risk for HCV since the prevalence of the HCV in the general population in Greece was higher (> 2%)^[52].

Renal transplant patients

A study which conducted in Greece reports that the prevalence of HCV infection in renal transplant recipients is high. Furthermore, the prevalence seems to be proportional to the haemodialysis time before transplant and inversely proportional to the time after transplant^[53].

RISK FACTORS

Medical procedures

The risk of HCV transmission *via* medical procedures is very low, especially nowadays. The hospital stuff follows the safety measures and the common medical procedures are associated with low prevalence of HCV transmission^[26]. As far as the dental procedures and the surgeries, the rate of HCV infections is small (10% of reported HCV infections)^[11]. In the past, infection rates were higher due to the use of non-sterilized, multiple-use glass syringes for medical purposes and warm glass bottles for folk remedies (for congesting blood in the muscles)^[20]. In the majority of cases the poor sanitation and sterilization methods were the cause for the HCV transmission.

Perinatal transmission

HCV transmission from HCV-infected pregnant women to their children may occur during pregnancy, labor or postnatal period^[54]. Two Greek studies have demonstrated that 0.80%-1.95% of the pregnant women tested positive for anti-HCV antibodies, with the incidence presenting a linear increase over the passing years^[55,56]. This elevated rate of HCV infection in this subpopulation is due to the extremely high rates of seropositivity among pregnant refugees^[55,56]. Raptopoulou-Gigi *et al.*^[56] suggest that mother-to-child transmission rate is 6% when mother is viraemic, whereas no transmission is reported when the mother is non-viraemic^[54]. Except from viral load, HCV/HIV co-infection and intravenous drug use history are also important risk factors for vertical transmission^[54,56]. As reported by Syriopoulou *et al.*^[54] viral transmission from mother to child was not influenced by mother's age, mode of delivery, genotype or type of feeding.

Sexual transmission

Sexual transmission of HCV in general population remains controversial. Percent of 15 of seropositive individuals report at least one high risk sexual contact in the last six months^[26]. In addition, prevalence of anti-HCV antibodies among prostitutes is higher than prevalence in general population^[26,57]. On the other hand, studies from other countries assert that sexual transmission of HCV among monogamous heterosexual couples is unlikely to happen^[58,59] and the maximum incidence rate is 0.07%^[58]. However, there is no similar Greek study to evaluate the risk of sexual transmission of HCV among couples.

HCV ROLE IN CHRONIC LIVER DISEASE AND HCC

Chronic liver disease is closely associated with HCV infection in Greece comparable to that reported in most European countries. The majority of patients with parenterally transmitted acute NANB hepatitis and almost 2/3 of those with sporadic NANB CLD were found to be associated with chronic HCV infection. Generally, chronic HCV infection was the cause of the single aetiological factor or co-factor, for about 1/4 (23.7%) of Greek patients presenting with CLD of any aetiology. Therefore, chronic HCV infection ranked second after chronic HBV infection in the aetiology of CLD in Greece^[60].

The association of anti-HCV with HCC has been examined in several studies. HBV and HCV have an interacting role in the origin of hepatocellular carcinoma and in hepatocarcinogenesis which, in some patients, appears to be related to underlying replication of both viruses^[60].

HIV/HCV CO-INFECTION

In Greece, the rates of HIV/HCV co-infection have ranged from 8.2% to 13.8%^[61-63] and 8.1% of patients with HIV/HCV co-infection are homosexual^[61]. In IVDUs population, HIV/HCV co-infection rates were increased over the last years^[36]. In the majority of patients with co-infection, HCV (85.7%) viral load is very high^[62]. However, in cases of HIV/HCV co-infection anti-HCV antibodies titer may be reduced or undetectable, because of the immunodeficiency^[26].

REFUGEES

Immigrants who live in Greece develop higher rates of infectious diseases. Seropositivity rate for HCV is 2.3%^[11,64] and it concerns mainly the refugees from the ex-Union of Soviet Socialist Republics and Africa (*i.e.*, Egypt) and less the refugees from Albania^[9,25,65-67].

GENOTYPE DISTRIBUTION

Genotypes 1a, 1b, 3a and 4a appeared in the Greek population around 1965, 1958, 1975 and 1967 respectively^[29]. In addition, it is mentioned that during 1978-1990 genotype 3 increased rapidly, whereas the other subtypes increased slowly during 1960-1990^[29,68]. Recent studies demonstrate genotype distribution in Greece. According to many studies, genotype 1 is estimated to be 45%-47% and it constitutes the prevalent genotype in Greece, followed by genotype 3^[4,11,68]. The incidence of the remainder genotypes is genotype 4 (13%), genotype 2 (9%), genotype 5 (1.2%) and genotype 6 (0%)^[4,11,69] (Figure 1). As far as subtypes are concerned, the most prevalent is 3a (32.9%), followed by 1b (26.8%) and 2a (6.1%).

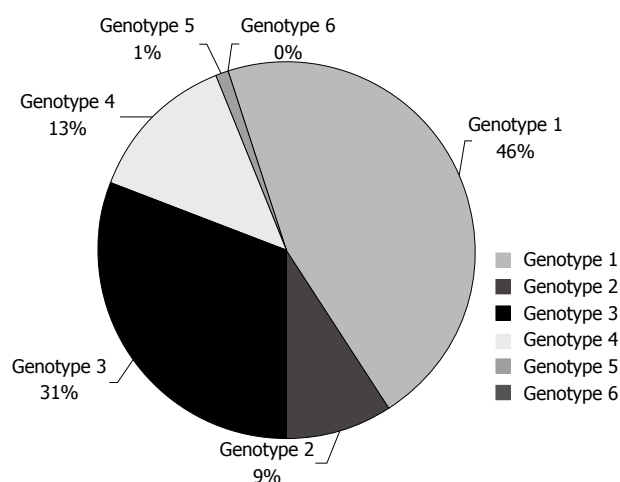


Figure 1 Genotype distribution in the Greek population.

Genotype distribution depends on various factors. Prevalence of genotypes differs between the genders. Genotype 1 is more prevalent in women, whereas genotype 3 is more common in men^[11,68]. In addition, genotype 1 is related to contaminated blood transfusion and genotype 3 is related to intravenous drug use^[4,11,29]. A recent study demonstrates that genotype 4 is more common among immigrants (18.8%) compared to Greeks (13.2%). These refugees are more likely to come from Egypt^[11]. Finally, Karatapanis *et al.*^[70] report a significantly high prevalence of genotype 5 in Rhodes, an island in South-East Greece compared to the rest regions of the country.

CONCLUSION

Hepatitis C constitutes a global health problem because of its adverse impact on quality of life and survival of patients. The definition of epidemiological patterns of infection will help to control the HCV transmission in Greece. Thus, more multicenter Greek studies are needed for the precise evaluation of prevalence of HCV infection in Greece.

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How significant is the association between metabolic syndrome and prevalence of colorectal neoplasia?

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Abstract

The incidence and prevalence of metabolic syndrome (MS) and colorectal cancer (CRC) has been rising in developed countries. The association between these two diseases has been widely studied and reported. Less evidence is available about the relationship between MS and CRC precancerous lesions (adenomatous polyps, adenomas). The aim of this paper is to present an overview of our scientific understanding of that topic and its implication in clinical practice. One of the principal goals of current CRC secondary prevention efforts is to detect and remove the precancerous lesions in individuals with an average CRC risk to prevent the development of invasive cancer. MS is not currently considered a high-risk CRC factor and is therefore not included in the guidelines of organized screening programs. However, in light of growing scientific evidence, the approach to patients with MS should be changed. Metabolic risk factors for the development of adenomas and cancers are the same - obesity, impaired glucose tolerance, dyslipidemia, hypertension, cardiovascular diseases and diabetes mellitus type 2. Therefore, the key issue in the near future is the development of a simple scoring system, easy to use in clinical practice, which would identify individuals with high metabolic risk of colorectal neoplasia and would be used for individual CRC secondary prevention strategies. Currently, such scoring systems have been published

based on Asian (Asia-Pacific Colorectal Screening Score; APCS) and Polish populations.

Key words: Metabolic syndrome; Diabetes mellitus type 2; Heart ischemic disease; Colorectal neoplasia

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Core tip: This article provides a review of our current understanding of the metabolic risk factors in the development of colorectal neoplasia and the scoring systems that may allow tailored secondary screening strategies. In addition, the preliminary results of a Czech multi-center prospective study investigating the relationship between metabolic syndrome and colorectal neoplasia are provided.

Suchanek S, Grega T, Ngo O, Vojtechova G, Majek O, Minarikova P, Brogyuk N, Bunganic B, Seifert B, Dusek L, Zavoral M. How significant is the association between metabolic syndrome and prevalence of colorectal neoplasia? *World J Gastroenterol* 2016; 22(36): 8103-8111 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i36/8103.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i36.8103>

INTRODUCTION

The incidence and prevalence of metabolic syndrome (MS) is rising in developed countries. The prevalence of MS based on National Cholesterol Education Program Adult Treatment Panel III criteria (NCEP-ATP III 2001) varies from 8% to 43% in men and from 7% to 56% in women worldwide^[1]. The Czech Republic is no exception; the prevalence of MS is estimated at 32% in men and 24% in women^[2]. MS reflects a combination of risk factors that often occur together and lead to the development of cardiovascular disease, type 2 diabetes mellitus and certain types of cancer, especially tumors of the gastrointestinal and genitourinary tracts^[3,4]. These risk factors include abdominal obesity, hyperglycemia, elevated blood pressure, elevated triglycerides and a low high-density lipoprotein (HDL) serum fraction of cholesterol^[5].

Colorectal cancer (CRC) with its tremendous population burden represents one of the greatest issues in contemporary health care around the world. The Czech Republic is among the countries with the highest incidence of CRC and related mortality in the world. Each year, there are 8000 new CRC cases and approximately 4000 individuals die from this disease^[6]. Central and Western European countries suffer from a long-term worldwide rise in the incidence and mortality of this disease. The Czech Republic currently has the fifth-highest incidence rate in Europe after Slovakia, Hungary, Denmark and the Netherlands^[7].

An organized screening program at the population level can successfully reduce the incidence and mortality of CRC^[8]. In the Czech Republic, the Czech National CRC Screening Program has been running since the year 2000, focusing on asymptomatic individuals older than 50 years with an average risk of CRC. Immunochemical fecal occult blood test (FOBT) is offered to asymptomatic individuals aged 50-54 years in one-year intervals. In the case of FOBT positivity, colonoscopy is performed. From the age of 55, every individual has the choice of either continuing examinations by FOBT every two years or undergoing a screening colonoscopy, which can be repeated in individuals with negative findings after ten years^[9,10]. In 2014, screening invitations addressed to the target population were introduced in the Czech Republic, effecting a transition from an opportunistic screening program to a population-based program. The total screening coverage of the target population in 2014 reached 31.5%, which was 4.6% higher than in 2013. Nevertheless, the total coverage of the Czech population by examination is below the optimum level. According to the European Guidelines, the total screening coverage of the target population should be at least 45% and optimally up to 65%^[11]. One of the most important barriers to screening is a lack of perceived risk of CRC among average-risk (AR) patients and primary care providers. New options for screening are therefore sought, such as targeted screening according to metabolic risk^[12]. To establish a targeted screening strategy for CRC, it is essential to define high-risk (HR) factors that are associated with colorectal neoplasia.

METABOLIC RISK AND COLORECTAL NEOPLASIA

Available evidence from epidemiologic investigations and clinical studies supports the hypothesis that MS may be an important etiologic risk factor for the development and progression of certain types of cancer, especially CRC^[13]. Studies on the association between MS and the risk of colorectal neoplasia are affected by the methodology (cohort vs case-control vs cross-sectional studies), cancer site (colon vs rectum), territory (United States vs Europe vs Asia), study quality, and the definition of MS. The main issue in the investigation of this association is the combination of multiple components of the MS. According to a harmonized definition from 2009, MS is present when any three of the following conditions are present: High waist circumference (≥ 102 cm in men, ≥ 88 cm in women), elevated triglycerides ≥ 1.7 mmol/L (≥ 150 mg/dL), reduced HDL cholesterol < 1 mmol/L (< 40 mg/dL) in men; < 1.3 mmol/L (< 50 mg/dL) in women, elevated blood pressure (systolic ≥ 130 mmHg, diastolic ≥ 85 mmHg) or serum glucose level ≥ 5.6 mmol/L (≥ 100 mg/dL)^[14].

OBESITY AND COLORECTAL NEOPLASIA

Obesity is associated with chronic low-grade inflammation due to the production of pro-inflammatory cytokines, such as tumor necrosis factor- α and interleukin-6, which lead to the secretion of acute phase proteins such as C-reactive protein^[15]. This chronic inflammatory process can result in a positive association between obesity and CRC risk. Additionally, a large number of newly discovered adipokines, such as leptin, adiponectin and resistin, are considered to be potential mediators of obesity in cancer development^[16].

The most common definition of obesity is a body mass index (BMI) of 30 kg/m² or greater. A large number of studies have reported an association between high BMI and CRC. For example, a study conducted by the American Cancer Society^[17] demonstrated that the relative risk (RR) of CRC death associated with high BMI (above 30 kg/m²) was 1.75 for men and 1.25 for women compared to individuals with a BMI below 25 kg/m². The association between CRC risk and BMI is stronger for cancers located in the distal colon than in other locations^[18]. BMI is also related to a higher risk of colon polyps or adenomas especially in the male population^[19]. The results of a large meta-analysis, including 70000 CRC cases, also indicate that obesity is directly and independently associated with CRC^[20]. Individuals with a BMI \geq 30 kg/m² have a approximately 20% greater risk of developing CRC than individuals of normal weight (BMI < 25 kg/m²). For every 2 kg/m² increase in BMI, the risk of developing CRC increased by 7%. Similarly, a 2-cm increase in waist circumference, a measure of central obesity, was associated with a 4% greater risk of CRC. These findings are similar to the results of another prospective meta-analysis conducted by Larsson in 2007^[21]. Those data also indicated a sex difference in the strength of the association; the risk of developing CRC was 30% higher in obese men than in obese women. A recent systematic review by Ning *et al.*^[22] found predominantly positive associations in the studies with an average RR for CRC of 1.18 (95%CI: 1.14-1.21) with a 5 unit higher BMI. The association was significantly ($P = 0.02$) stronger for colon cancer (RR = 1.21, 95%CI: 1.17-1.26) than for rectal cancer (RR = 1.11, 95%CI: 1.06-1.16). The association was significantly ($P = 0.001$) stronger in men (RR = 1.25, 95%CI: 1.2-1.3) than in women (RR = 1.12, 95%CI: 1.06-1.16).

The reason for a sex difference in the association between obesity and CRC may be partly explained by different hormonal levels (especially estrogen) in women. The Women's Health Initiative Estrogen Plus Progestin trial suggested that hormone replacement therapy reduced invasive CRC incidence by 44%^[23]. However, a follow-up study of women using estrogen alone did not reveal a reduction in CRC incidence^[24].

Another reason for a sex difference may be the differential distribution of adipose tissue. Several studies have demonstrated that central obesity is an

independent risk factor of colorectal neoplasia and represents a higher risk factor for CRC than BMI^[25]. Adipose tissue distribution can be assessed by the measurement of waist and hip circumferences. Current guidelines suggest a waist circumference of 102 cm in men and 88 cm in women as being the cut-off points for abdominal obesity that is associated with an increased risk of morbidity. Findings from the European Prospective Investigation into Cancer and Nutrition indicated that central (abdominal) obesity is an equally strong risk factor for CRC in both genders, whereas body weight and BMI are associated with CRC risk in men but not in women^[26]. The adipose tissue distribution (waist circumference or waist-to-hip ratio) was more associated with CRC risk than BMI^[27].

IMPAIRED GLUCOSE TOLERANCE AND COLORECTAL NEOPLASIA

Glucose intolerance or an elevated serum concentration of insulin is thought to be a risk factor for the development of CRC^[28]. This association is based on the hypothesis that hyperinsulinemia promotes colon carcinogenesis. Insulin resistance leads to a compensatory increase in insulin secretion, and this hyperinsulinemia may lead to increased levels of free insulin-like growth factor-1, an antiapoptotic and mitogenic factor that decreases cell death and enhances cell growth^[29]. Prospective epidemiological evidence has shown that hyperactivation of the insulin pathway leads to colon cancerogenesis by mitogenic and pro-angiogenic proliferation^[30]. According to the Netherland observational population-based cohort study, type 2 diabetes was associated with a moderately increased risk of CRC^[4]. Similarly, a large ten-year prospective cohort study in Korea found that serum glucose concentration was strongly associated with colon cancer^[31]. Yuhara *et al.*^[32] reported a significantly higher risk of colon cancer in diabetes patients (RR = 1.38, 95%CI: 1.26-1.51) compared to controls.

A recent Italian meta-analysis shows a 29% increased risk for CRC among individuals with dysglycemia, a condition including states of impaired fasting glucose and impaired glucose tolerance, or overt diabetes mellitus type 2^[27]. Another meta-analysis^[33] of 29 eligible studies confirmed these estimates indicating an increased risk of CRC in type 2 diabetes (RR = 1.29 for men and 1.34 for women).

DYSLIPIDEMIA AND COLORECTAL NEOPLASIA

The mechanism by which hypertriglyceridemia promotes CRC is unknown. One of the hypotheses cites the effect of secondary bile salts in the colon. Bile salts are increased in patients with high fat intake. An increase of secondary bile salts in the colon may have a carcinogenic effect on the colonocytes^[34].

The results of studies that have examined the association between serum triglyceride and HDL-cholesterol, components of MS, and the risk of CRC are inconsistent^[35,36]. Some recent prospective studies reported a significant association between high triglyceride levels and colon cancer in men^[37] or rectal cancer in both sexes^[38]. In a European case-control study, high concentrations of serum HDL were associated with a decreased risk of colon, but not rectal, cancer^[39]. The European Prospective case control study with a cohort of more than 520000 participants from 10 western European countries showed that high concentrations of serum HDL are associated with a decreased risk of colon cancer^[39]. However, the most recent Italian meta-analysis found nonsignificant or borderline positive associations between higher values of serum triglycerides and lower values of HDL cholesterol and CRC risk^[27]. Currently, the finding of an association between colorectal adenoma formation and hypertriglyceridemia seems more robust than for CRC^[40].

HYPERTENSION AND COLORECTAL NEOPLASIA

Few studies have examined the association between hypertension and the risk of CRC. In some studies, hypertension was found to be a predictor for colorectal adenoma formation^[41], but other studies reported conflicting findings^[25]. Ahmed *et al*^[42] assessed the data from a multi-center prospective cohort study. Hypertensive patients were found to have a 35% greater incidence of CRC compared to normotensive patients. However, these findings were not confirmed by a large Finnish study of male smokers that found no association between hypertension and CRC^[43]. A recent retrospective study from Taiwan observed that hypertension was a key predictor for recurrent colorectal adenoma^[44]. Interestingly, in a Japanese prospective study, the use of antihypertensive drugs was found to be a potential risk factor for the formation of colorectal polyps^[45]. This risk increased with the greater use of antihypertensive drugs. Nevertheless, the current general consensus is that hypertension does not contribute to an increased risk of developing CRC^[40].

CARDIOVASCULAR RISK AND COLORECTAL NEOPLASIA

With regards to the burden of cardiovascular and cancer morbidity, respective mortality, the same risk factors were identified and the same regimen recommendations were formulated, including the principles of healthy nutrition, physical exercise regimen, and quitting smoking and harmful alcohol consumption^[46]. Based upon our current knowledge and experience, the assumption may be expressed

that the higher risk of cardiovascular mortality also accompanies a higher risk of cancers, particularly CRC^[47]. Chan *et al*^[48] have shown a strong association between colorectal neoplasia and overt coronary heart disease (CHD). They have found that the prevalence of colorectal neoplasia was greater in patients with CHD in a population undergoing coronary angiography. Yang *et al*^[47] have found that the prevalence of colorectal neoplasia was greater in subjects with low-grade coronary atherosclerosis or significant CHD detected by coronary computed tomography angiography. Individuals with overt CHD are burdened with an increased risk of complications in endoscopic examination due to anticoagulation therapy and comorbidities. For targeted screening, it is important to identify individuals who are at high risk of CHD. However, in the literature, there is a lack of information on the risk of colorectal neoplasia in patients who are at a high risk of developing CHD. Lee *et al*^[49] have assessed the prevalence of colorectal neoplasia in South Korean patients who are at high risk for CHD by considering their Framingham risk score (FRS). They found an increased prevalence of advanced colorectal neoplasia in subjects with a high FRS $\geq 10\%$. Similar results came from a cross-sectional study from Turkey^[50], whose authors showed a significantly increased risk for colorectal neoplasia in patients who were at a high risk for CHD determined by ultrasound measurements of carotid intima media thickness (≥ 1.0 mm), flow-mediated dilation ($< 10\%$) and calculated FRS ($> 20\%$). According to these results, screening for CRC may be recommended for individuals who are at a high risk of developing CHD.

MS AND COLORECTAL NEOPLASIA

There is epidemiologic evidence to support the claim that subjects with MS are at increased risk of developing CRC. Obesity and hyperglycemia are key components of MS and CRC. According to these results, it appears that MS is also associated with a higher incidence of adenomas. This fact is especially important in CRC screening when identifying individuals at a higher risk for CRC in the general population. However, there are few studies demonstrating any association between MS and the risk of colorectal adenomas in European countries^[26,27,51]. In the Portuguese prospective study, MS was associated with an increased prevalence of adenomas (43% vs 25%, $P = 0.004$) and CRC (13% vs 5%, $P = 0.027$), compared to patients without MS^[51]. The literature conclusions suggest that different components of the MS have an additive effect on the development of CRC by acting through a variety of pathophysiologic pathways^[41]. However, an Italian meta-analysis that evaluated the influence of individual components of the MS observed that the increased risk of CRC was not greater than the sum of its parts, while the most common risk factors

Table 1 Asia-Pacific Colorectal Screening scoring system (adapted from Wang *et al.*^[53])

Risk factor	Criterion	Point
Age (yr)	< 50	0
	50-69	1
	≥ 70	2
Gender	Female	0
	Male	1
Immediate family member with colorectal cancer	No	0
	Yes	1
Smoking status	No smoking history	0
	Current or former smoker	1

Table 2 Risk score for advanced neoplasia (adapted from Segnan *et al.*^[11])

Risk factor	Category	Point
Age (yr)	40-49	0
	50-54	1
	55-59	2
	60-66	3
	> 66	3
Sex	Female	0
	Male	2
Family history	None	0
	1 first-degree relative ≥ 60 years old	1
	1 first-degree relative < 60 years old	2
	2 first-degree relatives	2
Smoking, pack years	None	0
	< 10	0
	10-19	1
	≥ 20	1
Body mass index (kg/m ²)	< 25	0
	25-29	0
	≥ 30	1 - Female 0 - Male

associated with MS were dysglycemia and/or high waist (≥ 88 cm in women and ≥ 102 cm in men)^[27].

ASIA-PACIFIC COLORECTAL SCREENING SCORING SYSTEM

One possibility for improving the effectiveness of screening for CRC can be observed in the scoring of the likelihood of colorectal neoplasia in the target population. In 2011, the Asia-Pacific working group for CRC screening developed the Asia-Pacific colorectal screening scoring system (APCS)^[52]. They conducted a prospective, cross-sectional and multi-center study of tertiary hospitals in 11 Asian cities, including 2752 asymptomatic individuals who underwent a screening colonoscopy. The main objective was to determine the clinical risk score predictive of colorectal advanced neoplasia in Asian asymptomatic individuals to prioritize CRC screening. The APCS scoring system uses the age, gender, family history of CRC, and smoking history to calculate the scores (Table 1). Individuals

are categorized into three groups according to these scores: The (AR = 0-1), moderate-risk (MR = 2-3), and (HR = 4-7) groups. In this study, patients in the MR and HR groups had a 2.6-fold (95%CI: 1.1-6.0) and 4.3-fold (95%CI: 1.8-10.3) higher rate of advanced neoplasia, respectively, than patients in the AR group. Therefore, a screening colonoscopy is recommended for Asian individuals in the HR group. The use of the APCS scoring system was further modified in the Chinese prospective study^[53]. They assessed the utility of the APCS system and the presence of MS components and found that in cases with obesity, the colorectal tumor detection rate significantly increased (59.5% vs 19.2% for the MR/HR group without obesity, $P < 0.01$). Utilization of the APCS scoring system in the Western population was examined in an Australian study by Corte *et al.*^[54]. APCS predicts the colonic findings in a Western population to a greater extent than in Asians, independent of the symptoms.

EUROPEAN COLORECTAL SCREENING SCORING SYSTEM

The risk score for predicting advanced neoplasia in Caucasian individuals developed by Kaminski *et al.*^[12] was established based upon a cross-sectional analysis of database records for patients aged 40-66 who entered a national primary colonoscopy-based CRC screening program in Poland in the year 2007. Candidate predictors of advanced neoplasia were obtained using a questionnaire and included age, sex, BMI, family history of CRC in first-degree relatives, diabetes, smoking history and aspirin use. The authors showed that independent risk factors for advanced colorectal neoplasia were age, sex, family history of CRC, cigarette smoking ($P < 0.001$ for these four factors), and BMI ($P = 0.033$). Based on these results they developed a scoring system that estimated the probability of detecting advanced neoplasia in the validation set, from 1.32% for patients scoring a 0 to 19.12% for patients scoring a 7-8 (Table 2).

However, a recently published Canadian work showed that the risk index for advanced neoplasia using age, sex, family history of CRC, smoking history and BMI, as derived by Ruco *et al.*^[55], was less predictive of advanced neoplasia in the population of screening age in North America.

CZECH PILOT STUDY

To study the relationship between metabolic risk and colorectal neoplasia, a multi-center prospective study was performed in the Czech Republic from 2012-2015. Eight endoscopy centers, 32 general practitioners and 24 diabetology practices participated in this project. All data were collected and analyzed by the Institute of Biostatistics and Analyses of Masaryk University in Brno.

Table 3 Comparison of non-advanced adenoma and colorectal neoplasia in the target and control groups

	Target group (<i>n</i> = 726)	Control group (<i>n</i> = 774)	OR, 95%CI (<i>P</i> value) ¹
Adenoma, <i>n</i>	346	270	1.2, 0.9-1.5
(%, 95%CI)	(48%, 44%-51%)	(35%, 32%-38%)	(0.18)
Advanced adenoma, <i>n</i>	131	66	1.8, 1.2-2.5
(%, 95%CI)	(18%, 15%-21%)	(9%, 7%-11%)	(< 0.01)
Cancer, <i>n</i>	11	11	0.7, 0.3-1.6
(%, 95%CI)	(2%, 1%-3%)	(1%, 1%-3%)	(0.35)

¹Adjusted comparison by logistic regression - including age, sex and previous FIT+ (fecal immunochemical test positivity).

The inclusion criteria were as follows: Asymptomatic individuals aged 45-75 years, individuals with an average risk of colorectal neoplasia (no personal or family medical history of colorectal neoplasia, no CRC symptoms, such as weight loss, enterorrhagia, or anemia), individuals scheduled for preventive (FOBT positive colonoscopy) or individuals aged 55 years or older (screening colonoscopy). The patients with a high risk of colorectal neoplasia (patients with familial hereditary syndromes of CRC or polyposis) were excluded.

Before the colonoscopy, blood samples were taken (complete blood count, coagulation, biochemical analyses including glucose, and lipid profile). After the colonoscopy, the anthropometric (weight, height, and waistline) and blood-pressure measurements were obtained, and the patients filled in a questionnaire about their personal and family medical history, lifestyle, medication and details of their FOBT examination (if performed).

Based on the collected data, the individuals were divided into a target group (patients with diabetes mellitus type 2 and/or cardiovascular risk) and a control group. The diagnosis of diabetes mellitus type 2 was assessed by glucose and glycated hemoglobin serum levels and confirmed by the oral glucose tolerance test. Cardiovascular risk was determined according to the SCORE project (Systematic COronary Risk Evaluation)^[56], based on four criteria: Sex, smoking habits, systolic blood pressure and serum cholesterol level. Patients with a SCORE level > 10 were included in the target group.

Colonoscopy examinations were performed mainly under conscious sedation after regular bowel cleansing. Colorectal neoplasia was defined based upon the presence of advanced adenomatous polyps (size > 10 mm and/or presence of villous component and/or high grade dysplasia) or cancer.

In this study, 2071 individuals were enrolled, and the first statistical analysis of 1500 records has already been completed. The main aim of the analyses was to compare the prevalence of colorectal neoplasia and non-advanced adenomatous polyps (adenomas) in both groups.

As the main finding, a significantly higher prevalence of advanced adenomas was observed in the target

group (18%, 95%CI: 15%-21%) compared to the control group (9%, 95%CI: 7%-11%); the OR was 1.8, and *P* = 0.002.

Similarly, the prevalence of all adenomas was higher in the target group (48%, 95%CI: 44%-51%) than in the control group (35%, 95%CI: 32%-38%); the OR was 1.2, but the difference was not statistically significant (*P* = 0.179). The prevalence of cancer was the same in both groups. Complete results are stated in Table 3.

Another aim of this study was to identify individuals who are considered to be at an average risk of developing colorectal neoplasia according to the current Czech guidelines, but because of their metabolic risk, should be managed similarly to patients at high risk of colorectal neoplasia (patients with a personal history of colonic neoplasia, *etc.*). A more comprehensive multivariate analysis, similar to that in the Polish study^[12], is planned once the data from all individuals are available.

As a first step towards this goal, a more detailed evaluation of the target group has already been performed by dividing the target group into three groups: Diabetes mellitus type 2 only; cardiovascular risk only; and a combination of these two risk factors. It appears that the individuals with cardiovascular risk only had a higher prevalence of both non-advanced adenomas (51%, 95%CI: 46%-56%, *P* = 0.327) and advanced adenomas (22%, 95%CI: 18%-26%, *P* = 0.049), compared to the other two groups (Table 4). Advanced adenomas were more likely in patients aged 65-75 years. This finding is in agreement with results of the work of Brenner *et al*^[57], which has shown that age is the most important risk factor for CRC.

As a conclusion of these preliminary results, individualized CRC screening should be considered in individuals aged 65-75 years with a SCORE ≥ 10.

CONCLUSION

The MS shows increasing prevalence worldwide. It has been shown that the strongest risk factors are central obesity and hyperglycemia in relation to CRC. Furthermore, cardiovascular risk is directly associated with the risk of colorectal neoplasia.

These observations should be reflected in future

Table 4 Prevalence of non-advanced adenoma and colorectal neoplasia within the target group

	Combination of DM2 and SCORE ≥ 10 ($n = 157$)	SCORE ≥ 10 ($n = 413$)	DM2 ($n = 156$)	<i>P</i> value ¹
Adenoma, <i>n</i>	69	211	66	0.33
(%, 95%CI)	(44%, 36%-52%)	(51%, 46%-56%)	(42%, 34%-50%)	
Advanced adenoma, <i>n</i>	25	89	17	< 0.05
(%, 95%CI)	(16%, 11%-23%)	(22%, 18%-26%)	(11%, 6%-17%)	
Cancer, <i>n</i>	3	5	3	0.56
(%, 95%CI)	(2%, 0%-5%)	(1%, 0%-3%)	(2%, 0%-6%)	

¹*P* value was obtained using a likelihood ratio test. The comparison models were adjusted for age, sex and previous FIT+ (fecal immunochemical test positivity). DM2: Type 2 diabetes mellitus; SCORE: Systematic COronary Risk Evaluation.

preventive strategies. While preventing and controlling the components of the MS could be important for the prevention of CRC, patients with the MS (including high cardiovascular risk) would probably benefit from tailored CRC screening.

A modified APCS scoring system and Risk Score for advanced neoplasia formulated by the Polish group can aid in identifying CRC HR subgroups in the general population.

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Iron and non-alcoholic fatty liver disease

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Abstract

The mechanisms that promote liver injury in non-

alcoholic fatty liver disease (NAFLD) are yet to be thoroughly elucidated. As such, effective treatment strategies are lacking and novel therapeutic targets are required. Iron has been widely implicated in the pathogenesis of NAFLD and represents a potential target for treatment. Relationships between serum ferritin concentration and NAFLD are noted in a majority of studies, although serum ferritin is an imprecise measure of iron loading. Numerous mechanisms for a pathogenic role of hepatic iron in NAFLD have been demonstrated in animal and cell culture models. However, the human data linking hepatic iron to liver injury in NAFLD is less clear, with seemingly conflicting evidence, supporting either an effect of iron in hepatocytes or within reticulo-endothelial cells. Adipose tissue has emerged as a key site at which iron may have a pathogenic role in NAFLD. Evidence for this comes indirectly from studies that have evaluated the role of adipose tissue iron with respect to insulin resistance. Adding further complexity, multiple strands of evidence support an effect of NAFLD itself on iron metabolism. In this review, we summarise the human and basic science data that has evaluated the role of iron in NAFLD pathogenesis.

Key words: Iron; Fatty liver; Liver steatosis; Insulin resistance; Steatohepatitis; Diabetes mellitus; Adipose tissue

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Core tip: Iron represents a potential therapeutic target for the treatment of non-alcoholic fatty liver disease (NAFLD). There are extensive data that link iron and disease pathogenesis in human studies as well as animal and cell culture models. Studies have predominantly focussed on the role of hepatic iron, although recently adipose tissue has emerged as a site at which iron may promote insulin resistance. In this review, we summarize the human and basic science data that have evaluated the role of iron in NAFLD pathogenesis.

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INTRODUCTION

The worldwide epidemic of obesity has led to a disturbing rise in the incidence of non-alcoholic fatty liver disease (NAFLD) and its complications^[1,2]. NAFLD, regarded as the "hepatic manifestation of the metabolic syndrome", is now estimated to affect one billion individuals worldwide^[1]. Non-alcoholic steatohepatitis (NASH), the aggressive form of the disease, can lead to cirrhosis and liver failure^[3,4]. Indeed, NASH is predicted to soon become the predominant cause of advanced liver disease in the developed world^[5] and the leading indication for liver transplantation^[4]. NAFLD has also been increasingly recognised as an independent risk factor for the development of type II diabetes mellitus, cardiovascular disease and hepatocellular carcinoma, the latter of which may occur even in non-cirrhotic individuals^[3,6,7]. The factors that predispose patients to the development of steatohepatitis and fibrosis in NAFLD are not well understood and effective treatment strategies are lacking^[8].

There is evidence that a modest degree of iron overload is associated with more advanced liver injury in NAFLD, although the mechanisms by which this might occur remain unclear^[9,10]. A syndrome of increased hepatic iron in conjunction with the metabolic syndrome is commonly observed and has been termed dysmetabolic iron overload syndrome^[9,11].

To date, the majority of studies have focussed mainly on the role of hepatic iron and mutations in the *HFE* gene, the gene mutated in type 1 hereditary hemochromatosis. Recently, however, it has become increasingly evident, that adipose tissue iron plays an important role in the pathogenesis of insulin resistance and therefore possibly NAFLD^[12,13].

In this review, the potential involvement of iron in NAFLD pathogenesis is explored using the available data from human studies, as well as animal and cell culture models. In addition, the counterview that implicates NAFLD itself in the dysregulation of iron metabolism is outlined.

HUMAN IRON HOMEOSTASIS

Iron is an essential nutrient required for erythropoiesis and multiple cellular metabolic functions^[14,15]. An excess of iron is also, however, a potent cause of cellular injury from oxidative stress due to the generation of reactive oxygen species by the Fenton reaction^[16]. Under usual conditions, intracellular

protection from iron-induced oxidative stress is facilitated by sequestration of iron within ferritin^[14].

Total body iron homeostasis is achieved predominantly by regulation of iron release from duodenal enterocytes and macrophages by the hormone hepcidin^[15,17,18]. Predominantly produced by hepatocytes, hepcidin binds the enterocyte basal membrane iron transporter, ferroportin, causing its internalisation and eventual degradation, thus reducing iron release from duodenal enterocytes and other cells^[15,18]. Ferroportin has been shown to be highly expressed in enterocytes, reticuloendothelial cells, and more recently, in adipocytes^[15,19]. Thus, hepcidin regulates systemic iron balance by reducing intestinal iron absorption^[15].

An understanding of the regulation of hepcidin (*HAMP*) gene expression has come about from studying human subjects with various forms of hereditary hemochromatosis, and by analysis of gene knockout rodent models. Hepcidin is regulated by many factors, including erythropoiesis, iron status, intracellular oxygen tension and inflammation^[18].

Pathologic states of iron overload often lead to saturation of serum iron transporter, transferrin. As a result, serum levels of toxic non-transferrin bound iron (NTBI) rise. NTBI is readily absorbed by tissues such as the liver and cardiac muscle^[18]. Tissue iron overload with NTBI results in increased oxidative stress and lipid peroxidation, leading to organ dysfunction. The common causes of iron overload include hereditary hemochromatosis, iron loading anemias (such as thalassemia) and parenteral iron overload from multiple blood transfusions^[18].

INSULIN RESISTANCE AND THE PATHOGENESIS OF NAFLD

It has become evident that insulin resistance is associated with a more subtle degree of iron overload than is seen in hereditary hemochromatosis and thalassemia^[9,10,12]. This is important as insulin resistance is central to the pathogenesis of NAFLD^[3,20]. The presence of abdominal obesity and accompanying insulin resistance provide fertile conditions for the development of NAFLD. Indeed, NAFLD is often considered as the hepatic manifestation of insulin resistance and the metabolic syndrome^[3]. Central obesity is associated with adipose tissue dysfunction, characterised by infiltration of adipose tissue with macrophages^[21]. Dysfunctional adipose tissue produces adipokines that promote the development of insulin resistance^[12]. The key sites of insulin action and resistance are the liver, skeletal muscle and adipose tissue^[22]. In adipose tissue itself, insulin resistance potentiates lipolysis of triglycerides by hormone sensitive lipase^[23]. This generates the majority of free fatty acid flux to the liver in NAFLD^[24]. Insulin resistance in skeletal muscle leads to reduced uptake of glucose, whereas in the liver, insulin resistance enhances gluconeogenesis^[25]. The

resultant compensatory hyperinsulinemia and relative hyperglycemia promote hepatic *de novo* lipogenesis and cholesterol synthesis and reduced catabolism of free fatty acid by oxidation^[3].

Increased hepatic free fatty acid flux resulting from this dysregulation of hepatic lipid metabolism and more importantly by adipose tissue lipolysis, appears to be central to the pathogenesis of steatohepatitis *via* direct lipotoxicity^[3,26,27]. A number of other mechanisms have been well demonstrated to be responsible for not only the development of steatohepatitis, but also steatosis itself. These mechanisms include dysregulated adipokine production^[28,29], abnormal bile acid signalling^[30], cytokine mediated effects^[31], in particular as a result of increased gut cell permeability and TLR-4 receptor activation^[32], endoplasmic reticulum stress^[33,34] and oxidative stress^[31,35]. Hepatocellular injury promotes cell death and steatohepatitis through a combination of apoptosis and cell necrosis^[3]. These mechanisms also contribute to hepatic stellate cell activation and resultant development of hepatic fibrosis^[36].

IRON AND INSULIN RESISTANCE

The association between hyperferritinemia, insulin resistance and type II diabetes is compelling. There is an increased prevalence of type II diabetes associated with two common iron overload conditions, HFE-hereditary hemochromatosis (HH) and β -thalassemia major^[12]. HH can lead to β -cell pancreatic loss and type I diabetes, but whether HH causes type II diabetes by unmasking insulin resistance through pancreatic β -cell loss or by causing insulin resistance itself remains controversial^[12]. Animal data suggest that insulin sensitivity is enhanced in HH, but it has been difficult to tease out the relative contributions of β -cell loss and insulin resistance in human studies^[12,37]. The case of β -thalassemia major is more clear, with evidence suggesting that both β -cell loss and insulin resistance are at play^[12].

In those who have neither hereditary hemochromatosis nor another cause of overt iron overload such as thalassemia, the evidence for a pathogenic role of iron is also strong. In the National Health and Nutritional Education Survey (NHANES), 9486 US adults were studied^[38]. The odds ratios for developing diabetes in those with elevated serum ferritin levels were high at 3.61 for women and 4.94 for men^[38]. A further analysis of the NHANES cohort revealed that even after accounting for other factors such as age, race, alcohol consumption and C-reactive protein (CRP) levels, elevated serum ferritin concentration still accounted for a two-fold increase in the risk of the metabolic syndrome^[38]. The risk of diabetes itself, has been shown to be strongly linked to serum ferritin concentration in healthy women, even within the normal range of ferritin^[39]. In 2012, the European Prospective Investigation in Cancer and Nutrition (EPIC)-Potsdam study followed 27548 European adults

for 7 years^[40]. In this time, 849 subjects developed type II diabetes. Serum ferritin concentration in the highest vs lowest quintile had a relative risk (RR) of 1.73 for the development of diabetes. This observation was made after adjusting for multiple variables including age, sex, body mass index, waist circumference, sports activity, education, occupational activity, alcohol, liver function test parameters, high sensitivity CRP (hsCRP), adiponectin, high density lipoprotein (HDL) and serum triglyceride concentration^[40].

A recent review of 43 studies further supported these findings^[41]. In this meta-analysis, the cohorts with the highest and lowest quartile of serum ferritin concentration were compared. The multivariable adjusted RR for the presence of diabetes was 1.91. This finding was consistent after including only studies that adjusted for inflammation (mostly hsCRP), RR 1.67. This related to a serum ferritin that was 43.54 ng/mL higher in type II diabetics compared to controls. Studies assessing the relationship between type II diabetes and transferrin saturation have yielded conflicting results^[41-43].

The persistence of association between serum ferritin concentration and type II diabetes after correction for hsCRP implies that inflammation alone does not entirely explain the association between hyperferritinemia and diabetes. However, it might be argued that even hsCRP may not reflect subtle degrees of inflammation as strongly as serum ferritin concentration.

SERUM FERRITIN CONCENTRATION AND NAFLD

The association between hyperferritinemia and histologic markers of liver injury in NAFLD is reasonably strong. In 2004, Bugianesi *et al.*^[44] found that serum ferritin concentration is not associated with hepatic iron concentration in NAFLD, but is a marker of severe histologic damage. Kowdley *et al.*^[45] demonstrated in the large NASH Clinical Research Network (CRN) cohort of 628 patients that a serum ferritin concentration greater than 1.5 times the upper limit of normal was independently associated with advanced fibrosis and increased NAFLD activity score. Sumida *et al.*^[46] have demonstrated the utility of incorporating serum ferritin into a clinical scoring system to predict steatohepatitis in Japanese patients with NAFLD.

However, other studies have not found such a clear association^[47,48]. Notably, Valenti *et al.*^[47] showed in an Italian cohort of 587 patients with NAFLD that serum ferritin concentration did not predict fibrosis stage > 1, although the proportion of patients with fibrosis stage > 1 in this cohort was relatively small. As would be expected, serum ferritin concentration was higher in the patients who had hepatic iron staining than those who did not, but those with non-parenchymal iron had much higher ferritin values (606 μ g/L) than those with hepatocellular iron (serum ferritin 354 μ g/L) $P < 0.0001$.

This might suggest that macrophage iron can cause hyperferritinemia either by direct release of ferritin or cytokine-mediated stimulation of ferritin release by other cells. An earlier study by Chitturi *et al.*^[49] of 93 patients with NASH, 33% of whom had advanced fibrosis, found that serum ferritin concentration was not an independent predictor of advanced fibrosis.

In a large prospective population-based study from South Korea, 2410 healthy men aged 30 to 59 without sonographic evidence of steatosis were followed for 7545.9 person years^[50]. Of these, 586 (24.3%) patients developed ultrasonographically detectable fatty liver. Baseline serum ferritin concentration was found to be a strong predictor of steatosis. This evidence is notable as it demonstrates an association early in the disease suggesting that the process that elevates serum ferritin concentration is contributing to NAFLD pathogenesis very early in the disease and pre-dates the development of steatosis. This implies that the ferritin association with NAFLD is not simply a result of NAFLD itself causing hyperferritinemia. Moreover, the results might tend to suggest that the link between hyperferritinemia and NAFLD could be explained by insulin resistance.

The strengths of these studies lie in the large numbers of individuals studied. However, serum ferritin concentration is an imprecise surrogate for body iron stores and its associations with both NAFLD and, type II diabetes are clearly not enough to attribute causality with respect to iron in either of these conditions.

HEPATIC IRON AND NAFLD

The role of hepatic iron in NAFLD pathogenesis has largely focussed on the generation of oxidative stress by iron. Given that oxidative stress is an established key component of NASH pathogenesis^[31], a role for iron mediating liver injury in NAFLD *via* this mechanism has been well studied. In NASH, oxidative stress leads to cell death *via* depletion of ATP, NAD and glutathione, and by direct damage to DNA, lipids and proteins within hepatocytes^[31]. Furthermore, oxidative stress leads to an increase in the production of pro-inflammatory cytokines and a fibrogenic response^[31]. Not only does oxidative stress potentiate steatohepatitis, characterised by inflammation and cell death, it can also increase steatosis by preventing the secretion of very low density lipoprotein (VLDL) by causing increased degradation of apolipoprotein B100 (ApoB100)^[51]. In cultured primary rodent hepatocytes, the iron chelator desferrioxamine was able to restore ApoB100 and enhance VLDL export^[51].

Reduced oxidative stress has been observed in the livers of rats fed an iron-deficient diet and after phlebotomy^[52]. In a series of liver biopsies from patients with NAFLD, increased hepatic iron stores were found to be associated with increased lipid peroxidation^[53]. In humans, iron overload has been

shown to correlate with hepatic immunohistochemical staining for 7,8-dihydro-8-oxo-2' deoxyguanosine (8-oxodG), a product of oxidative damage to DNA^[54]. In this study, staining for 8-oxodG was significantly reduced with venesection^[54]. Patients with NASH have been shown to have elevated levels of serum thioredoxin, a marker of oxidative stress, which declined following venesection^[55]. In cultured AML-12 hepatocytes iron generated oxidative stress and led to impaired insulin signalling^[56].

Iron also appears to have a direct role in the activation of hepatic macrophages and hepatic stellate cells. In humans with NAFLD, reticulo-endothelial iron has been shown to be associated with apoptosis, indicated by increased serum cytokeratin-18 (CK-18) fragments and increased hepatic TUNEL staining of liver sections^[57]. *In vitro*, iron activates inflammatory signalling *via* hepatic macrophages^[58]. Recently, dietary iron loading in leptin-receptor deficient mice was found to lead to inflammasome and immune cell activation with hepatocellular ballooning^[59]. Furthermore, ferritin treatment of rat hepatic stellate cells has been shown to lead to a pro-inflammatory cascade by nuclear factor kappaB signalling^[60].

Iron may also contribute to liver injury in NAFLD by generating endoplasmic reticulum stress^[61]. In a mouse model of dietary iron overload and NAFLD, iron induced an unfolded protein response and endoplasmic reticulum stress^[61]. Additionally, hepatic iron loading in mice up-regulates cholesterol biosynthesis pathways and this has been proposed as an additional mechanism of iron-induced liver injury in NASH^[62]. The proposed mechanisms relating to hepatic iron in NAFLD pathogenesis are summarized in Table 1.

A number of studies have looked at the relationship between hepatic iron concentration (HIC) and liver injury in NAFLD. George *et al.*^[63] showed that HIC was associated with increased fibrosis in 51 patients with NASH. Three subsequent and similar studies, however, have failed to reproduce these results^[44,64,65]. Two much larger studies have looked at the association between hepatic iron (Perls') staining and liver histology in NAFLD with conflicting results. In a study of 587 Italian patients with NAFLD, Valenti *et al.*^[47] found that hepatocellular rather than reticulo-endothelial iron was associated with 1.7 fold increased risk of significant fibrosis compared to those without iron staining. Reticulo-endothelial iron was found to have a trend towards an association with a lower risk of significant fibrosis. Nelson *et al.*^[66], however, found seemingly contradictory results, with reticulo-endothelial iron being associated with greater risk of advanced fibrosis, lobular inflammation and hepatocellular ballooning in the US cohort of 849 patients enrolled in the NASH CRN database. In this study, the mean NAFLD Activity Score (NAS)^[67] was 4.8 in the reticulo-endothelial iron staining group compared to 4.0 in the hepatocellular iron staining group. The exact reasons for this

Table 1 Proposed mechanisms for the involvement of iron in non-alcoholic fatty liver disease pathogenesis

Site	Mechanism
Hepatic iron	Oxidative Stress ^[31,53-57]
	Reduced VLDL export ^[51]
	Macrophage activation ^[57-59]
	Stellate cell activation ^[60]
	Endoplasmic reticulum stress ^[61]
	Increased cholesterol synthesis ^[62]
Adipose tissue iron	Reduced adiponectin ^[19,73,74]
	Reduced leptin ^[76]
	Increased resistin ^[75]
	Increased lipolysis ^[77,78]

discrepancy between these two large well-designed studies is unclear, although it is noted that there were some differences between the Italian and US cohorts including the frequency of steatohepatitis and beta-globin mutations^[9].

One might argue, however, that the sum of the human data indicates that if hepatic iron does promote liver injury in NAFLD, then its effect is likely to be relatively small.

ADIPOSE TISSUE IRON AND INSULIN RESISTANCE

In recent years, there has been increasing recognition of the role of adipose tissue dysfunction in the development of insulin resistance and NAFLD^[28]. Adipose tissue is undoubtedly a significant endocrine organ^[68]. It is comprised of adipocytes (fat cells), a mixture of cells categorised as the stromal-vascular fraction including reticuloendothelial cells, predominantly macrophages^[68]. Central obesity and the metabolic syndrome are characterised by infiltration of bone marrow-derived macrophages into adipose tissue^[21,69]. Macrophage accumulation in adipose tissue is associated with obesity and the development of NAFLD^[21,28]. A loss of regulatory T-cells and an increase in CD8+ effector T-cells characterises visceral adipose tissue in insulin resistance^[28,70,71]. The net effect of this adipose tissue infiltration with immune cells is a state of systemic low grade inflammation that is mediated by a number of adipose tissue cytokines, termed adipokines^[68]. Ectopic fat, such as omental (visceral) and epicardial or mediastinal fat, is dysfunctional tissue that is more likely to undergo inflammation^[72]. In the case of visceral fat, this inflammation is particularly problematic with regards to liver physiology due to the direct transfer of adipokines to the liver *via* the portal vein^[29].

Adipokines are polypeptides that are expressed significantly in adipose tissue in a regulated manner^[29]. Of these, a number of important macrophage derived adipokines appear to play an important role in the development of NAFLD. Both tumour necrosis factor alpha and interleukin-6 have a pro-inflammatory

role that may contribute directly to liver pathology in an endocrine fashion, and also *via* paracrine mechanisms that influence the production of other adipokines from adipocytes^[28]. Adipokines produced by adipocytes which have been shown to influence NAFLD pathogenesis include adiponectin, leptin, resistin, suppressor of cytokine signalling-3 and secreted frizzled related protein 5^[28,29].

Adipose tissue has been proposed as a site at which iron may have a major pathogenic role in NASH^[9]. Unfortunately, to our knowledge, direct human data reporting iron concentrations in visceral adipose tissue and its significance in disease are lacking and this area represents both a target for future research and a technical challenge.

Evidence for the role of adipose tissue iron in NAFLD pathogenesis mainly comes indirectly from the association between adipocyte iron and insulin resistance. In 2012, Gabrielsen *et al.*^[19] demonstrated that adipocyte iron reduced adiponectin gene expression, serum adiponectin levels and glucose tolerance in an adipocyte-specific *Ferroportin* knockout mouse model. Using the novel Ap2-Cre: Fpn^{fl/fl} model they were able to selectively load iron into adipocytes. The model was developed following the observation that adipocytes are high expressers of ferroportin^[19]. Using cultured pre-adipocytes (3T3-L1 cells) and chromatin immunoprecipitation analysis, iron was shown to alter acetylation and binding of the forkhead transcription factor Foxo1 to adiponectin gene promoter binding sites. In a human arm of the same study, they were able to demonstrate an inverse correlation between serum ferritin concentration and adiponectin that was independent of inflammation. This observation has subsequently been replicated in 492 Dutch individuals with risk factors for type II diabetes^[73]. Moreover, in obese patients undergoing bariatric surgery, two gene expression markers of increased adipocyte iron loading: increased hepcidin gene (*HAMP*) mRNA expression and decreased transferrin receptor 1 (*Tfr1*) mRNA expression were associated with reduced quantities of *Adipoq* (adiponectin gene) mRNA^[74].

Iron-mediated dysregulation of two other adipokines has been demonstrated in rodent models. Dongiovanni *et al.*^[75] have shown that dietary iron loading in mice leads to increased expression of resistin *via* SOCS-3 which are mediators of insulin resistance. Recently, data from mouse and 3T3-L1 cell culture models found that iron down-regulates the expression of the appetite-suppressing adipokine, leptin - a hormone strongly implicated in NAFLD pathogenesis^[29,76]. Intriguingly, this may help explain the symptom of anorexia in iron deficiency, although the significance of these findings in NAFLD is uncertain.

Adipose tissue iron has been shown to directly enhance lipolysis in isolated rat adipocytes and cultured 3T3-L1 cells^[77,78]. As adipose tissue is the predominant source of free fatty acid flux to the liver^[24], this is potentially a very important mechanism

of adipose tissue iron action in NAFLD, although these findings are yet to be demonstrated in animal models or humans. Potential mechanisms relating to adipose tissue iron in NAFLD pathogenesis are summarized in Table 1.

In summary, iron has been increasingly recognised as a regulator of adipose tissue function. Evidence supports a role for iron in the regulation of adipose tissue inflammation, adipokine regulation and adipose tissue lipolysis. At present, most of the evidence supports a role for adipose tissue iron in the pathogenesis of insulin resistance and type II diabetes, although clearly these mechanisms may be highly relevant in NAFLD.

IRON-RELATED GENETIC POLYMORPHISMS IN NAFLD PATHOGENESIS

The most common inherited disorder affecting the hepcidin-ferroportin axis is type I hereditary hemochromatosis^[16,18]. This usually results from homozygous p.C282Y mutation of *HFE* (*HFE*-hemochromatosis)^[79]. The additional insult of NAFLD acts as a co-factor for the development of liver injury in C282Y homozygotes with hereditary hemochromatosis^[80]. In non-hemochromatotics, the broader significance of *HFE* gene mutations as co-factors in the pathogenesis of NAFLD has received intense interest in recent years. The two most significant *HFE* mutations in Caucasian populations are the p.C282Y and p.H63D mutations^[18].

Heterozygosity for the C282Y mutation is found in approximately 10%-11% of individuals in Caucasian populations^[81,82]. C282Y heterozygosity is associated with a mild increase in serum iron markers, but not with overt hemochromatosis^[82].

Many studies have looked at the association between *HFE* gene mutations and the incidence of NAFLD, but with conflicting results. These studies may have been limited by inadequate statistical power and heterogeneity of the cohorts. In 2011, Hernaez *et al.*^[83] published the results of a meta-analysis of 13 case-control studies specifically aimed at determining the association between *HFE* gene mutations and NAFLD. In contrast to a previous meta-analysis by Ellervik *et al.*^[84], they found no association between the C282Y/C282Y genotype and NAFLD. Similarly the presence of neither the C282Y mutation nor the H63D mutation resulted in an increased risk of NAFLD in Caucasians. In a sub-analysis of three studies of non-Caucasians, an association was found between the presence of the H63D mutation and the presence of NAFLD^[83].

A limitation of the meta-analysis, as noted by its authors, is that it was not able to determine whether *HFE* gene mutations might have a disease modifying role in subjects after they have developed NAFLD^[83]. This study appears to show that *HFE* gene mutations are generally no more common in subjects with NAFLD

than in those without, however, the investigators were unable to determine whether those patients with NAFLD and *HFE* gene mutations are more likely to develop steatohepatitis and progressive liver injury than those without mutations.

The issue concerning the effect of heterozygous mutations in progression to NASH was highlighted by an analysis of *HFE* mutations within the NASH CRN cohort^[85]. This is a well-defined cohort of patients with biopsy proven NAFLD. Subjects with the H63D mutation had higher steatosis grades and NAS than their wild-type controls. However, those NAFLD patients with C282Y mutations had lower rates of hepatocyte ballooning and steatohepatitis.

Our group has previously shown that mice with homozygous knockout of the *Hfe* gene develop severe steatosis, steatohepatitis and early fibrosis when fed a high fat diet, whereas wild-type mice develop mild steatosis and no steatohepatitis or fibrosis when fed the same diet^[86]. *Hfe* null mice had only modest increases in HIC, and it was proposed that the increased histologic injury seen in these animals may have been due to the lack of *HFE* protein rather than iron overload *per se*. *Hfe* null mice demonstrated dysregulated hepatic lipid metabolism with increased transcription of genes associated with *de novo* lipogenesis and reduced transcription of those associated with fatty acid oxidation^[86].

A number of other non-*HFE* iron-loading polymorphisms have been proposed as modulators of NAFLD pathogenesis^[9,87]. Of these, the A736V polymorphism of the *Trans-membrane protease serine-6* (*TMPRSS6*) gene has been studied in patients with NAFLD. The *TMPRSS6* gene encodes for matriptase-2, an enzyme responsible for hemojuvelin cleavage that inhibits the bone morphogenetic protein-6 pathway, thus reducing hepcidin expression and increasing duodenal iron absorption^[18,87]. Of 216 Italian patients with NAFLD, 38% had the AA genotype, 47% AV and, 15% VV^[87]. The VV genotype is associated with increased hepcidin expression and reduced iron loading and in this study was associated with a trend ($P = 0.05$) towards a reduction in hepatocyte ballooning^[87].

In summary, human and animal model data support a role for a co-toxic liver injury in the setting of hereditary hemochromatosis and NAFLD. Other more mild iron loading phenotypes such as heterozygous *HFE* gene mutations and polymorphisms of *TMPRSS6* may have disease modifying roles in NAFLD, although their effect is likely to be small.

CLINICAL TRIALS OF IRON REDUCTION THERAPY

Although associations of modest iron overload with NAFLD and diabetes appear reasonably well established, causality is difficult to determine using these studies alone. The most useful information with which

to more directly assess causality comes from human studies that have assessed the response to iron removal by venesection.

Venesection has been shown to improve glucose tolerance in healthy individuals and improve insulin sensitivity in type II diabetics with a high serum ferritin concentration^[88,89]. Moreover, in patients with the metabolic syndrome, venesection has been shown to improve metabolic syndrome parameters, including reduced blood pressure, blood glucose, glycosylated hemoglobin (HbA1C) and low-density lipoprotein/high density lipoprotein (LDL/HDL) ratio^[90]. In patients with NAFLD and carbohydrate intolerance, venesection to near iron deficiency (decrease in serum ferritin from 299 ± 41 $\mu\text{g/L}$ to 15 ± 1 $\mu\text{g/L}$) not only improved insulin sensitivity, as measured by fasting glucose, insulin and homeostatic model assessment-insulin resistance (HOMA-IR) score, but also improved serum alanine aminotransferase levels from 61 ± 5 U/L to 32 ± 2 U/L^[91].

Two randomised controlled trials investigating venesection efficacy in NAFLD have recently been published. In a study of 38 Italian patients with NAFLD and hyperferritinemia, participants were randomised to venesection versus no venesection with liver biopsy before and after treatment^[92]. Of the 38 enrolled participants, 21 underwent liver biopsy at the end of treatment. Despite the small numbers, histological improvement, defined by an improvement in NAS, was seen in 8 of 12 participants in the venesection group compared to 2 of 9 participants in the control group ($P = 0.04$)^[92].

The largest randomised study of venesection in NAFLD to date involved 74 Australian participants with NAFLD^[93]. These included patients with sonographically detected NAFLD and a wide range of serum ferritin concentration, including many within the normal range. Non-invasive assessment was performed to assess response to randomised therapy of either venesection with lifestyle advice versus lifestyle advice alone. There was no observed effect of venesection on hepatic steatosis determined by magnetic resonance imaging, serum ALT or CK-18 fragments. Somewhat surprisingly, there was also no effect on static and dynamic measures of glucose homeostasis including the HOMA-IR score and insulin sensitivity index^[93].

Overall, although there are promising results from small studies, venesection cannot currently be recommended as a suitable therapy for the majority of patients with NAFLD^[94]. However, whether there are sub-groups of non-hemochromatotic NAFLD patients with increased iron that would benefit from venesection, remains to be determined by further studies.

IRON METABOLISM IN NAFLD

So far, we have discussed the effect of iron on the pathogenesis of NAFLD and insulin resistance. It is also necessary to consider to what extent NAFLD and

associated conditions, such as insulin resistance and obesity, might themselves mediate iron metabolism.

Serum hepcidin levels are typically elevated in individuals with NASH^[95]. As this in itself fails to explain iron loading in NASH, one might consider that dysregulated iron metabolism occurs in NASH independently of hepcidin. In this regard, *Transferrin receptor-1 (Tfr1)* has been shown to be upregulated as a consequence of a high fat diet in mice which may lead to hepatocellular uptake in NAFLD despite already increased hepatocellular iron^[96]. Also, divalent metal transporter 1, which is responsible for import of iron from the duodenal lumen into enterocytes is up-regulated in patients with NASH, despite increased serum hepcidin^[97]. Another intriguing finding is that increased red cell fragility in response to a high fat diet in rabbits leads to increased erythrophagocytosis^[98]. This may explain increases in hepatic reticuloendothelial iron that have been observed in some NASH cohorts^[66].

It seems likely that elevated hepcidin in NASH is either a reflection of hepatocellular inflammation or simply that increased iron, which induces hepcidin, pre-dates the development of NASH. Indeed, hepcidin expression appears to be directly enhanced by insulin and down-regulated in the setting of insulin resistance, thus indicting a possible mechanism for iron loading as an early event in the pathogenesis of NAFLD and type II diabetes^[99]. Furthermore, it has been observed that hepcidin is expressed in white adipose tissue and is increased in obesity^[100]. Although the contribution of adipose tissue-derived hepcidin to the serum hepcidin pool is uncertain, this is another potential factor that may explain increased serum hepcidin in NASH. Further complexity in these relationships arises when one considers that iron deficiency has been shown to be associated with obesity and in women with obesity and NAFLD^[101,102]. Together, these findings suggest that the interaction between iron and lipid metabolism is multi-faceted. It seems that "just enough" but "not too much" iron may be critical in preventing dysfunctional lipid metabolism.

If one accepts a causal role for iron in NASH pathogenesis, then variations in dietary iron may explain much of the spectrum of iron loading in NASH. Although there is no specific evidence relating iron intake to NASH pathogenesis in humans, increased dietary iron, particularly from red meat, seems to predispose individuals to the development of insulin resistance and type II diabetes^[103-105].

CONCLUSION

In summary, there is considerable evidence that links increased iron stores with insulin resistance and NAFLD. This includes a number of studies that have identified serum ferritin concentration as a predictor of liver injury. Hepatic iron itself is attractive culprit for liver injury, although the cellular location of iron within the liver may vary between genetically distinct

populations. Increasingly, adipose tissue iron has been linked with adipose tissue dysfunction, including the dysregulation of adipokines, enhanced adipose tissue lipolysis and adipose tissue inflammation. These are plausible candidate mechanisms that may link adipose tissue iron to liver injury. However, assessment of adipose tissue iron concentrations in individuals with well characterised NAFLD remains a goal for future studies.

Iron-related genetic polymorphisms, such as those of the *HFE* gene, may contribute to NAFLD pathogenesis, although it would appear that, other than for individuals with hereditary hemochromatosis, the effect of these polymorphisms, is likely to be small. The complexity of these relationships between iron and NAFLD is further increased when one considers the possibility that NAFLD itself is likely to have a number of effects on iron metabolism.

Finally, venesection studies have offered a unique opportunity with which to assess causality of iron loading in the pathogenesis of NAFLD. The available data suggest that venesection is unsuitable as a general treatment for all patients with NAFLD. Therefore, the key for future human studies will be to determine whether a subset of patients with NAFLD can be identified that might still benefit from therapeutic manipulation of iron homeostasis.

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Inflammatory bowel disease in India - Past, present and future

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Abstract

There is rising incidence and prevalence of inflammatory bowel disease (IBD) in India topping the Southeast Asian (SEA) countries. The common genes implicated in disease pathogenesis in the West are not causal in Indian patients and the role of "hygiene hypothesis" is

unclear. There appears to be a North-South divide with more ulcerative colitis (UC) in north and Crohn's disease (CD) in south India. IBD in second generation Indian migrants to the West takes the early onset and more severe form of the West whereas it retains the nature of its country of origin in migrants to SEA countries. The clinical presentation is much like other SEA countries (similar age and sex profile, low positive family history and effect of smoking, roughly similar disease location, use of aminosaliclates for CD, low use of biologics and similar surgical rates) with some differences (higher incidence of inflammatory CD, lower perianal disease, higher use of aminosaliclates and azathioprine and lower current use of corticosteroids). UC presents more with extensive disease not paralleled in severity clinically or histologically, follows benign course with easy medical control and low incidence of fulminant disease, cancer, complications, and surgery. UC related colorectal cancer develop in an unpredictable manner with respect to disease duration and site questioning the validity of strict screening protocol. About a third of CD patients get antituberculosis drugs and a significant number presents with small intestinal bleed which is predominantly afflicted by aggressive inflammation. Biomarkers have inadequate diagnostic sensitivity and specificity for both. Pediatric IBD tends to be more severe than adult. Population based studies are needed to address the lacunae in epidemiology and definition of etiological factors. Newer biomarkers and advanced diagnostic techniques (in the field of gastrointestinal endoscopy, molecular pathology and genetics) needs to be developed for proper disease definition and treatment.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; India; Review

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Core tip: There is growing interest in inflammatory

bowel disease (IBD) in India due to its rising incidence. This review addresses the current state of knowledge on different aspects of Indian IBD patients like epidemiology, genetics, mechanisms, clinical presentations and treatment (compared to other south Asian countries) in the context of which future areas of research is highlighted. The disease is milder in India. Well-designed population based studies are needed. To address the obscure pathogenesis and uncertain disease course (behaviour, activity, treatment response, development of cancer and prognosis) studies on mechanisms, biomarkers, advanced endoscopic techniques need to be done to decrease the morbidity burden.

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INTRODUCTION

Inflammatory bowel disease (IBD) encompasses ulcerative colitis (UC) and Crohn's disease (CD), two chronic inflammatory disease of uncertain etiology affecting the gut. IBD started gaining special attention in India only after mid 1980s with wider availability of colonoscope. In the era before 1985, UC was often difficult to distinguish from more prevalent infectious colitis and CD was only reported on surgical specimens^[1] and even its existence was questioned^[2]. Clearly the studies had limitations as due to lack of equipments the extent and severity of bowel involvement, biopsy taking from proximal gut areas and diagnosis of superadded infections was difficult and treatment options were limited. The period 1986-2000 saw a resurgence of interest in IBD (especially UC) not only due to availability of better resources but also increased reporting of IBD in the 1st and 2nd generation Indian diasporas settled abroad. This period saw well described clinical studies on UC. The period 2000 - 2010 saw further spread of the speciality of gastroenterology all over India with availability of even better infrastructure especially technology to visualise the small gut (capsule and balloon endoscopes). This spurred an increase in number of well documented publications on CD (and intestinal tuberculosis which made comparison easy between the two). With increase in patient awareness, spending potential and availability of health insurance, access to specialised health care became a reality at least for a section of common people who otherwise could not have afforded it. This increased the number of patients available for detailed study. After 2010 with availability of better opportunities for research in basic science focus has been on genetic aspects, deeper insight into the disease mechanisms and development of new drugs.

EPIDEMIOLOGY

In North America and northern Europe (areas with highest IBD occurrence), the incidence of UC and CD ($6-12/10^5$ and $5-7/10^5$ population respectively) are much higher than southern Europe ($2-8/10^5$ and $0.1 - 4/10^5$ population respectively). IBD was traditionally thought to be of low occurrence in eastern Europe, Asia and Africa till recently. The temporal trend is variable in different geographic areas. In North America and northern Europe, UC initially increased from 1935 to 1964, then decreased till 1979 and thereafter have been stable. The incidence is increasing in southern Europe with a tendency to catch up with the north. CD started increasing from 1960 with plateauing after 1975 but recently have been rising all over the world^[3-5].

Recent reports show IBD to be rising in Asia albeit much less than Europe^[6]. In India the first case of CD was reported about 23 years after UC though in surgical specimen^[1]. Since then 2 epidemiologic studies have been conducted in north India on UC only^[7,8]. A house to house survey of 4796 houses including 21921 persons (> 14 years age) in Haryana state revealed 10 cases (5 each in both sexes) which gave a prevalence of $45.5/10^5$ population ($42.8/10^5$ for males and $48.6/10^5$ for females)^[7]. In a later study from the neighbouring state of Punjab^[8] where cluster sampling method was employed the crude incidence and prevalence of UC was found to be $6.02/10^5$ and $44.8/10^5$ population which was the highest in Asia but still less than that of North America and Europe. This was similar to the prevalence reported in the study 17 years earlier indicating stability over time. There have been no epidemiologic study from any other parts of India though the recent IBD task force data^[9] fulfils some of the gaps especially for CD. The IBD Task Force was set up in 2003 to collect data prospectively by questionnaire method from all over India. Participants were all qualified gastroenterologists from all corners of India. Of 1159 questionnaires analysed, UC: CD was 750:409 and region wise distribution were North 220 (148:72), East 159 (90:69), Central 255 (227:28), South 466 (235:231), West 59 (50:9). Thus CD was much higher in the south followed by east compared to other regions. Most of this data was however hospital based.

Other indirect evidence of prevalence comes also from hospital based series on diarrhoea, GI bleed and malabsorption which are the principle symptoms of IBD. In an early study in 1990 on patients attending mission hospitals in India, Nepal, Pakistan and Bangladesh, IBD was diagnosed in only 74 cases (UC 56, CD18) out of 12272 cases of bloody diarrhoea^[10]. Later three colonoscopic studies from north India found IBD in 19.3%^[11] and 5.5%^[12] cases of lower GI bleed and in 25% cases of chronic large bowel diarrhoea^[13].

With the advent of better radiologic and endoscopic modalities for visualising the small gut after 2000, CD is

being diagnosed with increasing frequency. Two series on the etiology of malabsorption in 275 and 94 cases from north India, found the diagnosis in 2% cases each^[14,15] whereas in a study of 124 cases from south India, 15.3% had CD^[16]. By small gut investigative modalities like capsule and balloon enteroscopy for symptoms of pain abdomen, malabsorption and obscure GI bleed (OGIB), CD was diagnosed in 12%-20% cases from south^[17-19], 11%-20% cases from east^[20-22]. Thus in India the general belief is that IBD is rising and a north-south divide is apparent between UC and CD. This divide may be the result of differences in genetics, hygiene and diet (see below).

RACE

The nature of IBD in Indians as a race is well highlighted by early epidemiologic studies in the diasporas and subsequently supported by Indian studies. In the early 90s, studies on South Asians in Leicestershire^[23,24] showed increased incidence of CD in South Asians ($2.4/10^5$ in Hindus, $3.4/10^5$ in Sikhs and $5.4/10^5$ in Muslims) and of UC in Sikhs ($16.5/10^5$) only but not in Europeans and other South Asians. The subjects had less number of complications and operations. In this context, it is interesting to note that the study from Punjab, a state with a Sikh majority in India, show relatively high incidence and prevalence of UC^[8]. Similarly, young Asian Indians born in Britain were more likely to have IBD by 26 years age compared to indigenous Europeans (OR = 6.1, CI: 2.14-17.33)^[25] and South Asians (Indians, Bangladeshis, Pakistanis) to have more pancolitis (63% vs 42.5%) and colonic CD (46.8%) but with less penetrating disease and need for surgery^[26].

Other studies on Indians in Canada^[27] and South Africa^[28] showed that the majority had pancolitis but clinically mild disease and Canadian born patients were younger than migrants.

In a very recent analysis of 30812 IBD patients from United States diagnosed between 2008 - 2013 (20308 with UC, 7706 with CD, and 2798 with indeterminate colitis), UC was more commonly associated with Indian and Jewish ethnicity and less with East Asian and Hispanic ethnicity. Similar patterns also applied to CD and to all types of IBD analyzed jointly^[29]. Studies in multiracial Asian countries also project similar findings. Indians in Fiji, Singapore and Malaysia constitute the majority of cases on UC compared to the local Chinese and other races, have more extensive disease yet least number of surgery^[30-33].

Thus there is a rapid rise in IBD among Indians settled abroad with younger age of onset and higher incidence of pancolitis (simulating the population of their country of settlement) but yet has less clinical severity, complications and surgery indicating a benign course. This is peculiar to Asian Indians as a race compared to Europeans and other Asians. Most

Indian studies (to be discussed below) also show similar features. A particular disease trait in a specific race might indicate a genetic link. The other point of note is that IBD in 2nd generation Indian diasporas settled in the West (Europe and North America) resemble their country of settlement, but in those settled in other Asian countries, it retains features of their country of origin. This might indicate some common environmental factor in Asian countries (diet, lifestyle) which has less effect on alteration of disease expression after migration.

GENETICS

Involvement of genes in the pathogenesis of a disease can be deciphered from the disease occurrence in family members, siblings and twins. The Indian IBD task force data showed positive family history of IBD in 2.9% cases only (UC 2.3% and CD 4.6%). In one study there was no association of HLA DR and DQ with CD^[34]. Studies on causative role of individual genes have only started recently in India especially with the discovery of single nucleotide polymorphisms (SNP) of 163 genes associated with IBD (at various points of its immune mechanism) in Caucasian populations (of whom *NOD2* gene shows the most definite association with disease phenotype). The association of different genes with IBD in India studied till now are shown in Figure 1^[35-55]. Multiple studies have negated the association of *NOD2* gene with CD in India^[52-54] but SNP 268Pro/Ser increased relative risk of UC (OR = 1.72, 1.14-2.52)^[51].

Few studies have demonstrated relation to UC phenotype but majority show increased risk or negative association only. Specific haplotypes of MDR1 (ABC B1) gene were well associated with early age (< 29 years) of disease onset, left sided disease and steroid response in UC^[48]. TNF alpha 863 AA genotype increased risk of both UC and CD, more for UC especially pancolitis. IL4 B2 carrier state was less in left sided colitis than proctosigmoiditis and absent in colonic CD^[42].

Of the SNPs associated with IBD in Caucasians detected by metanalysis of GWAS studies, only 5 of 59 index ones studied in North India were found significant showing limited replication in Indians^[44] and there was varying contribution to risk by the HLA region with 3 novel HLA independent risk loci for UC^[38]. HLA DRB1*0103 was absent while HLA DRB1*1502 was increased in Indian UC patients compared to controls and Europeans. There was no significant difference between the ethnic groups in *NOD 2* and *IL23* gene^[56]. Thus the common causative genes in Caucasian population are non-causal in India which could be due to differences in microbiome (the first line interact of *NOD 2* gene) or secondary factors like diet, pollution, use of antibiotics and proton pump inhibitors which differentially affects the phenotypic outcome either by themselves or by altering the microbiome.

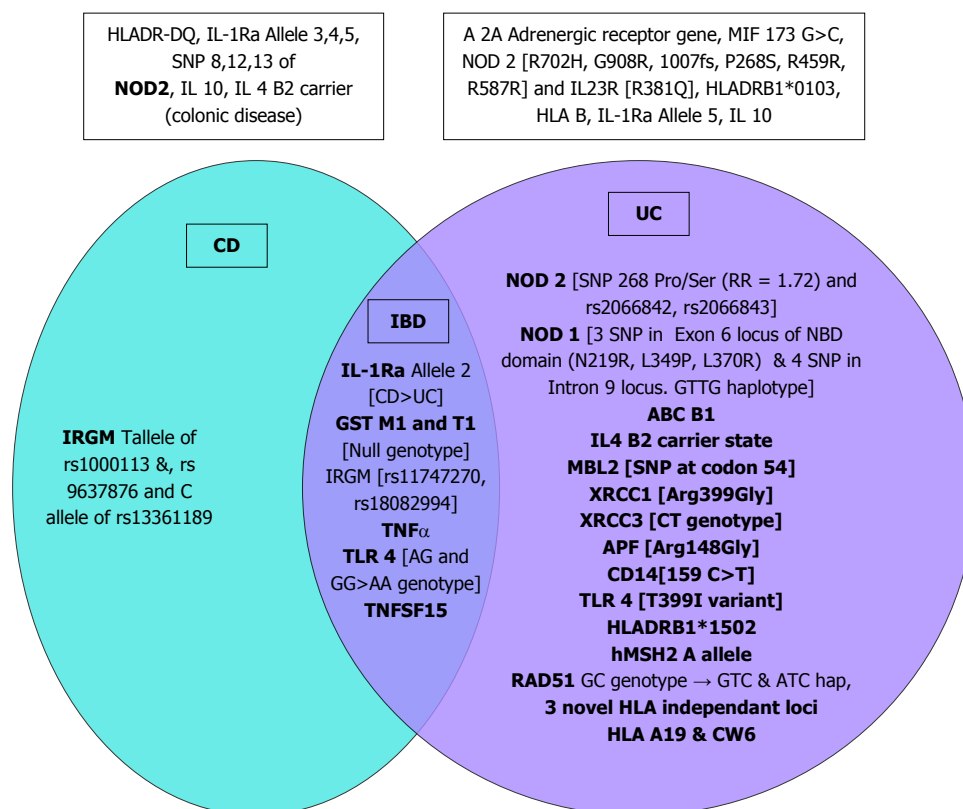


Figure 1 Association of different genes with inflammatory bowel disease, ulcerative colitis and Crohn's disease. Genes shown outside the Venn diagram were not associated. CD: Crohn's disease; IBD: Inflammatory bowel disease; UC: Ulcerative colitis.

A traditional thinking in India has been that of a genetic difference between the North and South Indians which is supported by 2 studies, one undertaken by the Indian Genome Variation Consortium^[57] and another from Singapore which compared the genes of two distinct migrant groups from India - the Indo-European language speaking Gujrati expatriates in United States and the Dravidian language speaking Tamil expatriates in Singapore^[58]. A genetic analysis of 132 individuals of 25 diverse groups in India predicted the existence of two ancestral groups in the pre-historic India: an "ancestral North Indian (ANI)", which shared genetic affinity with the populations of the Middle East, Central Asia and Europe (30% to 70%), and an "ancestral South Indian (ASI)", which has no relation with any population outside India *i.e.*, they were the indigenous population of India^[59]. The epidemiologic features of UC in North Indians (who are supposed to have originated from the above regions as Indo - Aryans) resembling the Caucasians may reflect this fact. The caste system in India maintained this genetic distinctness due to marriages within one's own caste. But the present-day Indian populations are an admixture of both ANI and ASI as shown in a most recent study^[60]. This is an obvious outcome of rapid migration of population from one region to another in India due to socioeconomic and political upheavals brought by rapid industrialisation with increase in intercaste marriages. This might obfuscate any further chance to

study the pristine genetics and their bearing on disease phenotype in these populations in future.

MECHANISM

IBD results from a strong genetic predisposition on which environmental factors act to produce dysregulated gut mucosal immune response to luminal antigens (the intestinal microbiota, normal or abnormal). The genetic findings discussed above may predispose, yet the increasing number of IBD cases reported within limited period of time in India cannot be explained totally by genomic changes but provide evidence for the importance of exposure to environmental factors in disease pathogenesis. Childhood infections, lack of breast feeding, exposure to helminths, smoking, repeated use of drugs like antibiotics and proton pump inhibitors, dietary and psychosocial factors have all been implicated in IBD pathogenesis and may act by altering this balance between the gut and microbiota either by themselves or by altering the latter^[61]. Childhood infections by producing tolerance of immunoregulatory T cells to external antigens early in life (immune conditioning) protects against IBD in adult life. This is exemplified by the (1) increasing incidence and earlier onset of IBD in children of Indian diasporas settled in cleaner and more hygienic environment of Europe and North America compared to their parents and counterparts settled in India as

Table 1 Environmental factors involved in inflammatory bowel disease causation in India

Study type (region)	Sample size	Factors significant on univariate analysis				Factors significant on multivariate analysis, OR (CI)	Factors not significant
		Ulcerative Colitis		Crohn's disease			
		Favours	Protects	Favours	Protects		
Case control ^[65] (south) ¹	200/200			Urban residence (birth/current)	Cattle in house compound (current)	Cattle in house compound (current) 0.57 (0.35-0.92)	Age, closed toilet in house, tooth cleanser use, pets in house (childhood/current), Cattle in house compound (childhood), appendicectomy, smoking, regular meat consumption
				Treated drinking water (childhood/current)	Regular fish consumption (> 1/wk)	Regular fish consumption (> 1/wk) 0.52 (0.33-0.80)	
				Piped water supply in house (childhood/current)		Treated drinking water (childhood/current) 1.59 (1.02-2.47)	
				Lactovegetarian			
				High socioeconomic score			
Case control ^[66] (north) ¹	513/188	Higher education (graduation and beyond)	Using private bed			Using private bed 0.25 (0.16-0.39)	Age, smoking, water source (municipal vs tubewell), illness in family, number of sibs
		Hand washing				Owning a pet 2.02 (1.14-3.59)	
		Having personal towel	RCA/flush type latrine			RCA latrine 0.29 (0.14-0.60)	
		Owning a pet				Flush type latrine 0.43 (0.23-0.82)	
		Death in family				Death in family 2.19 (1.58-4.07)	
Case control ^[67] (east)	50/50	Higher intake of refined sugar				Higher intake of refined sugar 1.78 (1.03-6.9)	Age, Sex, socioeconomic status, regular smoking and alcohol intake, rice, wheat, meat, fish, fried food, tea/coffee, green leafy vegetables ²
		Low intake of fruits				Low intake of fruits 0.28 (0.15-0.65)	

¹More females had disease than controls; ²Higher intake of green leafy vegetables ($P = 0.05$) tended towards protective association.

well as children of the local Caucasian population; and (2) increasing incidence of allergic and autoimmune diseases with decreasing childhood infection in Western epidemiologic studies^[62]. The increasing incidence of CD (over UC) was first observed in Western countries as the sanitation - hygiene started improving with disappearance of helminthic (which promote Th2 response protective against CD) and other infections which is now happening in developing countries. Other pointers to this association are the increasing CD incidence in urban vs rural setting, higher vs lower socioeconomic strata and in whites vs natives. In India the highest reporting of CD comes from Kerala, a south Indian state with the highest literacy rate and better hygiene^[63].

In a study from south India, T cell activation [increase in CD3(+) CD69(+) population] by hookworm antigen and interferon- γ ELISPOT responses to hookworm antigens were significantly higher in controls compared to CD patients^[64]. Other studies on environmental factors are summarised in Table 1^[65-67]. In the survey by the IBD Task Force, more number of CD cases had appendicectomy though rates of smoking and oral contraceptives intake were same as UC^[9]. While some of these findings are

similar (less effect of smoking, presence of hot water tap and flush toilet in childhood protective for UC) to other south east (SE) Asian countries but many are not (owning pet increase risk of UC, daily tea/coffee consumption has no effect on both UC and CD) and the effect of some have not been studied (breast fed > 12 mo, antibiotic use, daily physical activity, western diet)^[68]. Type of diet consumed varies widely in different regions of India. In general rice based diets are common in south and east (areas with more CD) whereas wheat is consumed more in north India (area with more UC). Studies are very few (listed in Table 1) and the findings need confirmation by more number of detailed community based cohort studies.

Microorganisms in pathogenesis

A number of studies have found altered number, type and location of colonic bacterial flora in IBD. Butyrate producing bacteria (*Clostridium coccides* and *leptum*) were significantly decreased in UC and differentially positive according to severity which increased with disease remission^[69,70]. A ten times increase in total bacterial count with increase in unusual anaerobes and facultative aerobes (*Proteobacteria*) and significant decrease in bacterial diversity from phylum to species

Table 2 Comparative clinical features of inflammatory bowel disease in different countries

Country	India ^[9]		South East Asia ^[81,82]		Australia ^[81,82]	
Factors	UC	CD	UC	CD	UC	CD
Median Age (yr)	38.5	35.9	42	34	42	34
Sex (M: F)	1.4:1	1.3:1	1.4:1	1.6:1	1:1.2	1:1.1
Smokers	21.3%	24.2%	6%	11%	8%	10%
Disease location						
L1		28.9%		31%		31%
L2		31.4%		24%		24%
L3		39.6%		45%		45%
L4		5.8%		5%		5%
Disease behaviour						
B1		76.8%		66%		88%
B2		18.8%		17%		10%
B3		4.4%		19%		2%
Perianal		6.9%		18%		12%
Disease extent						
Proctitis	18.3%		37%		32%	
Distal colitis	38.8%		32%		27%	
Extensive colitis	42.8%		31%		41%	
Treatment						
5ASA	89.7%	58.4%	68%	49%	86%	71%
Corticosteroids	29.1%	26.9%	21%	42%	50%	67%
Immunosuppressive	29.8%	62.9%	8%	35%	0	14%
Biologics	0	2.2%	1%	5%	0	0
Antibiotic	0	0	25%	19%	0	21%
Probiotics	6.1%	7.6%				
Surgery	4.0%	15.2%	1.6%	11.6%	5.9%	14.3%
Positive family history (%)	2.3%	4.6%	31%		17% ¹	
EIM						
Joints	33.3%	26.3%	13% ¹		3.6% ¹	
Backache	31.5%	36.4%	3.3[AS] ¹			
Skin	2.4%	3.4%	4.8% ¹		3.6% ¹	
Incidence per 10 ⁵ population	6.07% ^[8]		0.76%	0.54%	7.33%	14%

¹Present in overall inflammatory bowel disease population. EIM: Extraintestinal manifestations; AS: Ankylosing spondylitis; UC: Ulcerative colitis; CD: Crohn's disease.

was noted in colonic flora of severe UC compared to mild and moderate disease^[71]. Other studies found clear delineation in bacterial concentration in mucosal layer between predominant and subdominant genera between UC and CD indicating involvement of different subsets of bacteria in pathogenesis^[72] and more mucosal adherence of *E. coli* in the colon of CD patients^[73]. Other organisms that have been associated with exacerbations of UC (detected in stool in 17%-30% cases of moderate to severe disease^[74-76]) are CMV in 2.3%-15.8% cases^[74,77]. *C. difficile* (toxin detected in 3.4%-32%^[74,78-80]) and rarely Giardia, Strongyloides, hookworm, herpes simplex, *E. histolytica*, cryptosporidium and salmonella^[74,75]. The exact significance of these in disease pathogenesis is unclear. In CD pathogenesis the contribution of Mycobacterium paratuberculosis remains contentious and that of Mycobacterium tuberculosis is discussed below.

CLINICAL FEATURES

The chief clinical features of IBD in India (as per the ISG Task Force data representative of the whole country) is summarised in Table 2 along with those of other countries of SE Asia and Australia (which

approximates that of the West in incidence, female dominance and higher positive family history) for a comparative view^[9,81,82]. The chief similarities with SE Asia are UC more than CD, age profile (with unimodal peak for UC), male dominance, low percentage of positive family history, less effect of smoking on disease, similar disease location (except for lower proctitis and higher pancolitis in case of UC like Australia), low use of biologics, high use of 5ASA for CD and similar surgical rates (including low colectomy rates for UC). The issue of biologics vs surgery in refractory cases is a matter of debate and mostly depends on cost of hospital stay and medicines, availability of expertise and infrastructure, ability to deal with stoma care and proper follow up.

The chief differences are the higher incidence of extraintestinal manifestations (EIM), disease behaviour in CD (higher inflammatory and lower penetrating disease similar to Australia), much lower perianal disease, higher use of 5ASA (similar to Australia especially for UC) and azathioprine (AZP) (compared to both SE Asia and Australia), lower current use of corticosteroids (especially for CD), no use of antibiotics for CD and a possibility of medicine discontinuation (17.5% of UC and 6.9% of CD cases). The reason is

that in Indian patients steroids are used intermittently (either at induction or for flares, so 2/3rd have history of past use^[9]), is tapered quickly to be replaced by AZP along with 5ASA as most cases are mild to moderate in severity with easy control and only few are steroid resistant or dependant. A latest study on disease behaviour over long follow up upto 15 years showed that most patients had aggressive inflammatory disease at presentation (as is the point incidence in Task Force data) which decreased over time with simultaneous increase in penetrating disease whereas stricturing disease remained stable. This was independent of age or disease location^[83]. Another large series spanning 13 years showed that small intestinal CD more often followed aggressive course and needed surgical intervention^[84]. Regarding higher incidence of EIM it must be mentioned that studies are very heterogenous regarding the methods (and hence the extent and minuteness) by which they are searched for and often terms like arthralgia and backache have been synonymously with EIM without proper documentation of lesions.

It was believed in the 80s that UC is a mild disease in India, the outcome variables being shorter length of colonic involvement, low rate of complications, surgery and mortality. Though in recent times there is increasing presentation with extensive disease in majority, the disease still runs a relatively benign course as reflected by easy medical control, low incidence of fulminant disease, cancer and requirement for surgery even in the longrun. This general disease pattern in Indians is reflected even in recent series published from multiracial Asian countries (see above).

The other difference in CD cases with the West is the higher percentage of OGIB at presentation representing small intestinal bleed. This is likely due to increasing presentation at younger age with aggressive inflammatory type small intestinal disease. The earliest Indian series on OGIB where small gut was visualised only by barium studies and intraoperative endoscopy, CD was confirmed in 20%^[22]. Subsequent series using capsule endoscopy, single and double balloon enteroscopy for symptoms of pain abdomen, malabsorption and OGIB have respectively yielded CD in 0-38%, 0-100%, 0-19%, cases, overall being 11.3%-29.2%^[17-21].

BIOMARKERS IN IBD

(1) ANCA positivity has been reported in 3%-32% of UC^[35,85] and 3.8%-10% of CD^[86,87]; (2) fecal lactoferrin is also a good marker of UC activity (sensitivity 94%, specificity 100%) being higher in cases and more severe disease and decreased in parallel with improvement of Mayo severity score with treatment^[88]. It has also been suggested to reflect disease exacerbation caused by *C. difficile*^[80]; (3) fecal calprotectin level was found to be higher in UC than ileocolonic CD and correlate well with clinical (by

Truelove-Witts score) and also endoscopic severity (by Mayo score) but not disease extent. A level of 800 mcg/gm stool best differentiated active from inactive disease^[89]; (4) positivity of ASCA in CD has been reported overall in 30%-62%, IgA in 34%-38% and IgG in 38%-50% all higher than in controls. The corresponding figures for UC were 26%-40%, 28% and 24% respectively, but only IgG ASCA was significantly lower than in CD. ASCA also does not differentiate CD from ITB^[86,87]. Thus ASCA does not appear to have diagnostic value for CD strong enough to differentiate with certainty from UC and ITB as in the West. However more studies are needed to elucidate the exact role of these biomarkers in disease definition.

COLORECTAL CANCER IN UC

Only 5 series^[1,90-93] present data on UC related colorectal cancer (CRC) from India listed in Table 3. In all the disease duration was long and there was predominance of pancolitis yet low rates of CRC. How can these facts be reconciled? The answer may lie in the fact that most of the pancolitis cases are not clinically or histologically severe and can be well controlled with medicines. The control of inflammation stops the impetus for carcinogenetic changes. The other factor may be the background low sporadic CRC rates in Asia and India possibly related to yet unidentified protective genes. (These UC related CRC rates though much lower than in the West are still higher than the prevailing low sporadic CRC rates in India). To address these issues properly, medically compliant high risk UC cases needs to be followed up for development of polyps, low grade dysplasia (LGD), high grade dysplasia (HGD), dysplasia associated lesion or mass and CRC in the sequence followed by sporadic CRC. Unfortunately the duration of disease after which screening should start and the intervals at which colonoscopy is to be done are unsettled and has to be tailored according to local conditions (15%-20% of CRC cases develop at less than 10 years of disease duration). Recent studies have tried to address these issues. In the retrospective study, 5 (0.94%) developed CRC and 1 (0.19%) HGD over 25 years. Surveillance was done for 6 years. Of the 5 CRC, one each had LGD and HGD detected 5 and 3 years earlier respectively and another had LGD in rectum 1 year earlier which persisted while CRC developed in descending colon. First CRC developed after 10 years of disease. Six patients had LGD at different areas (single or multifocal) which either disappeared or did not progress in next 1 to 2.5 years follow up^[90]. It thus appears that development of CRC is unpredictable and may occur at site different from dysplastic areas even in synchronous manner^[94]. This suggests molecular and pathological heterogeneity during multiclonal origin of UC related CRC, where UC randomly damages multiple carcinogenetic genes in epithelial cells. This

Table 3 Comparative data on ulcerative colitis related colorectal cancer in different Indian series

Study type	Incidence of CRC	Time duration of FU in years	Presented with CRC	CRC developed at FU	Risk of CRC at time in years	Risk Factors	LGD	HGD	Detected on surveillance/ symptoms
Retrospective ^[91]	8/436 (1.8%)	12.1 (7-25)	4	4 (2/4 after 7, 8 yr of FU)		Pancolitis 6/8 (66.7%)	NM	NM	Symptoms
Retrospective ^[90]	5/532 (0.94%)	25	0	5 (in FU of 6 yr)	0 at 10, 2.3% (4.4% for pancolitis) at 20, 5.8% (10.2% for pancolitis) at > 20	Pancolitis, disease duration > 10 yr	2 progressed to CRC (1 after 5 yr, 1 at a different site after 1 yr). 6 did not progress in 1-2 1/2 yr	2 (1 CRC after 3 yr, another operated immediately)	2 CRC on routine surveillance
Partly Retrospective, partly prospective ^[1]	5/50 (10%), [0/21 (prospective), 5/19 (retrospective)]	9.35 (1-30), prospective 4.5 (1-12), retrospective 15 (8-30)	0	5 (1 after 10 yr, 4 after 20 yr)	0 at 10, 1% at 20, 7% at > 20	Disease duration > 10 yr	No surveillance	No surveillance	5 at symptoms
Prospective ^[93]	12/430 (2.8%)	Median 6 (1-39)	0	After median 18 yr from onset (6-27), 3 at 6, 6, 7 yr FU	Incidence density per 103 PYD was 2.3 at 10, 3.3 at 20, 7 at > 20	Pancolitis, longer disease duration	NM	NM	1 on surveillance after 11 yr/11 at symptoms, (unifocal in 10 and multifocal in 2)
Prospective surveillance study ^[92]	1/29 (3.4%)	10 (7.5-14.5)	0	1 (after 1 yr)		Pancolitis in 55%	5 at baseline, 3 new cases on surveillance	3 (1 CRC after 1 yr)	1 over 42 mo surveillance

CRC: Colorectal cancer; FU: Follow up; LGD: Low grade dysplasia; HGD: High grade dysplasia; PYD: Person years of disease duration; NM: Not mentioned.

also put question mark on the usefulness of strict screening protocol with respect to time interval and number of segmental biopsies. In another prospective study, only 29/41 (70.7%) eligible UC cases (55% pancolitis > 7 years and rest limited segment colitis > 10 years duration) could be enrolled for colonoscopic surveillance over 42 mo (done by magnification chromoendoscopy). Initial screening showed LGD in 5 (17.2%) and HGD in 3 (10.3%). Only 12 follow up colonoscopies could be done in 9 cases at intervals of 6-24 mo which detected 3 new LGD cases and adenocarcinoma in one HGD case^[92]. This study showed surveillance to be useful but acceptance and proper follow up to be suboptimal in India. Albeit these uncertainties of disease progression, cost effectiveness and acceptability of screening protocol, it seems reasonable for surveillance to start after 8-10 years in cases of pancolitis (10-12 years for limited colitis) and subsequent intervals determined by the findings at baseline of LGD (3-5 years), HGD (1-2 years) and also by frequency of relapses, quality of medical control and the expected disease duration at initial diagnosis.

In a study of culprit CRC genes, p53 mutation increased from 3.3% in low risk UC cases to 27.3% in high risk UC cases without CRC, to 50% in high risk UC cases with CRC and 33.3% in sporadic CRC. KRAS mutation was present only in presence of neoplasia but BRAF mutation was absent in all cases. Therefore the mutations are similar to the West except MSI^[95]. The same group also reports increasing number of copy number variations spanning multiple chromosomes to be associated with the progression of UC to CRC from low risk through high risk cases, dysplasia cases to CRC^[96] some of which are common and unique to both UC associated and sporadic CRC. These are present in 10 overlapping regions of a number of chromosomes (highest in 12p and 8q chromosome) and have overall accuracy of 29% and 54% for detecting these cancers respectively^[97].

DIFFERENTIATION OF CD FROM ITB

Indian CD patients are diagnosed after a average gap of 1 and 1/2 years from symptom onset mainly because of diagnostic confusion with ITB. The clinical,

serologic, radiologic, endoscopic, microbiological and histologic features which distinguishes ITB from CD are discussed at length in recent review^[98] and series^[99-101] and the reader is referred to these articles for detailed discussion. However in spite of such in depth theoretical differentiation, 36.7% receive antituberculosis drugs at some point in their disease course^[9]. This underscores the practical difficulty of differentiating the two in community setting where experts and advanced diagnostic facilities are not widely available. A recent attempt at ultrastructural and molecular differentiation can be seen *e.g.*, (1) claudin 2 expressed in the upper part of intercellular junction with maintained tight junction in ITB compared to CD where it is increased along the whole length of intercellular junction^[102]; (2) mesenchymal cell marker CD73 being expressed only in ITB granuloma but not CD granuloma^[103]; and (3) increased expression of growth related oncogene alpha mRNA in biopsy specimen and of IL17 in peripheral blood mononuclear cells of ITB cases compared to CD whereas increased IL 1, IL 6 and IL 8 in peripheral blood mononuclear cells and increased gamma interferon, TLR 5 and 9 and decreased RANTES in biopsy specimen of CD cases compared to ITB^[104].

The important questions are whether the two diseases can coexist throughout their course, or whether Mycobacterium tuberculosis initiate CD by producing altered gut mucosal immunity like other gut microbiota and more recently whether it can cause disease exacerbation in patients on immunosuppressives. It is now clear that a large portion of IBD risk loci are shared with other immune-mediated diseases, primary immunodeficiencies and mycobacterial disease, pointing towards common pathogenic mechanisms between different diseases. CD is associated with genes which code for proteins involved in autophagy and innate immunity highlighting the importance of defective processing of intracellular bacteria in its pathogenesis. It is intriguing to note that seven susceptibility loci for infection with Mycobacterium leprae, including NOD2, IL23R, RIPK2 and TNFSF15, have also been associated with CD though their final effects are in different directions. It remains to be investigated whether this genetic overlap signifies a true causative role for mycobacteria in CD, or rather represent the result of convergent evolutionary adaptations to different pathogens^[61]. A very recent report of intestinal tuberculosis diagnosed in a long standing case of CD on immunosuppressive and it to be causally associated with Takayasu disease is interesting enough to raise question^[105].

PEDIATRIC IBD

There are very few published series on the topic. Only very recently a multicentre questionnaire survey involving large number of pediatric cases both from north and south India have been published^[106] which

endorses the previous two small series only on CD from south India^[107,108]. Very interestingly the north south divide between UC and CD, clinical features, EIM and treatment results are all similar to their adult counterparts. Whereas the UC:CD ratio is 2:1 among north Indian patients, it is just the reverse among cases from south India. Pancolitis was the commonest mode of presentation in UC (70.9%) though clinically it was moderate in 84%. Eighty-eight percent required steroids of whom 43% needed AZP subsequently for maintenance in addition to 5ASA. Biologicals were used in 6 (0.8%) but 4 needed surgery. For CD, ileocolonic involvement was commonest (72.9% of whom 76.2% required AZP for maintenance) while upper GI and perianal modifiers were present in 18% each. Eighty-four percent needed steroids of whom 76.2% were maintained on 5-ASA + AZP and 12.2% were given infliximab. Twenty point four percent developed fistulae, stricture, and perforation of whom 8.2% needed surgery. The differences were (1) CD commoner than UC; (2) growth failure (76.2%, 40%) and anemia (64.7%, 43%) are common in CD, UC; (3) no gender difference in both UC and CD; (4) CD was more severe, 76.2% had moderately severe disease and 19.6 % had fulminant disease; and (5) more than 80% of both UC and CD needed steroids. Similar facts have been noticed recently in pediatric South Asian population in America also^[109,110].

CONCLUSION

IBD occurs much more commonly in India than previously thought with incidence and prevalence topping Asian countries and approaching the lower limit of that of North Europe and America which might have involvement of both genetic (inheritance from Caucasian origin) and environmental (causal for the recent quick rise in incidence) factors. The common genes implicated in pathogenesis in the West are not causal in Indian patients and the causal environmental factors needs larger community based cohort studies to be pinpointed. There appears to be a North-South divide with UC being higher in north than south India while the situation is reverse for CD though population based studies are lacking from south India. IBD in 2nd generation Indian diasporas settled in the West resemble their country of settlement but in those settled in other SE Asian countries retains features of their country of origin. The clinical presentation is much like other SE Asian countries (similar age and sex profile, low positive family history and effect of smoking, roughly similar disease location, high use of 5ASA for CD, low use of biologics and similar surgical rates) with some differences (higher incidence of inflammatory CD with much lower perianal disease, higher use of 5ASA and AZP and lower current use of corticosteroids). UC presents more with extensive disease not paralleled in severity clinically or histologically, follows benign course with easy medical control and low incidence of fulminant disease,

cancer, complications, and surgery. This specific disease behaviour in Indians as a race might also be genetically determined with environmental modifiers. UC related CRC develop in an unpredictable manner with respect to disease duration, site and dysplastic areas questioning the validity of strict screening protocol. The diagnostic confusion of CD with ITB is still prevalent and in spite of multiple investigations for differentiation, about a third of CD patients still get antituberculosis drugs. A significant number of CD cases presents with small intestinal bleed where the disease predominantly affects with aggressive inflammation. A significant number require surgery in the longrun. Biomarkers have inadequate diagnostic sensitivity and specificity for both. Pediatric IBD tends to be more severe than adult.

FUTURE

Presently there is scarcity of well-designed population based studies from India to authenticate the apparent temporal rise and the regional differences in incidence and prevalence of IBD. These have to be undertaken from different parts of the country, especially from south where CD seems to be highly prevalent compared to north. Ideal epidemiologic study should ensure universal accessibility to health care, a population based sample, standardised case definition, relevant database/registries with appropriate validation and prospective data collection^[6]. Most published studies are hospital based which are likely to underestimate incidence as more severe disease is only seen. But there might be true increase in incidence due to rapid industrialisation, urbanisation and change in diet and lifestyle. With rapid mixing of population all over India forced by political and socioeconomic factors, the genetic distinctness and hence disease phenotype is also likely to change and may further obfuscate the unclear present scenario.

Fortunately IBD has not yet attained the dimension of a pressing public health problem and hence it is difficult to visualise active participation of government in addressing its problems in near future. Yet as people live long with the disease, the morbidity burden is going to rise and patients will mandate special support in the longrun from all specialities involved in the management of this complex disease (gastroenterologist, surgeon, radiologist, nutritionist, stoma care specialist, pathologist) which will skyrocket cost of treatment. The government, national and state societies (ISG, SGEI) and other related foundations (CCFI) can cooperate to establish proper registries, conduct well designed epidemiologic studies, set up patient care centres (IBD centres) in different states where patients needing lifelong special care can get support at affordable cost. Case definition and treatment protocol needs to be standardised, widely circulated and adherence ensured. It will also be the responsibility of physicians to refer appropriate cases

early to IBD centres. Newer biomarkers and diagnostic modalities needs to be made generally available for proper diagnosis in all aspects of the disease. Treatment options (both existing and newer) needs to be tested rigorously in randomised controlled trials (RCT) especially costly drugs like biologics, ciclosporin and others to define appropriate use of each (especially in view of the milder disease in India) to avoid unnecessary spending by patients. In a low prevalence country like India, the usefulness and feasibility of screening for CRC surveillance has to be carefully addressed. The disease duration after which the surveillance should start, the frequency of follow up, type of endoscopy to be used (magnification, chromo, confocal endomicroscopy) and the number and colonic areas of biopsy has to be fixed. Considering the low patient number, options will be difficult to test in RCTs.

Lastly some of the problems facing IBD management all over the world, not the least in India, has to be addressed also. These are (1) an obscure pathogenesis which prohibit formulation of uniform diagnostic and therapeutic strategies; (2) uncertain disease course including behaviour, activity, treatment response, development of cancer and prognosis; and (3) differential diagnosis of unclassified IBD colitis.

IBD results from an interplay of host genetic factors and environmental factors with the multiple microbial ecosystems present in different areas of the human bowel (both luminal and mucosal). Mucosal surface of the intestinal tract (which is continuously exposed to a large number of microorganisms) produce a diverse array of antimicrobial proteins, some constitutively and some in response to invasion of pathogens or enteric microbiota into the mucosal barrier. An interplay of diverse pathogenetic pathways (both stimulatory and regulatory) first produce an array of mucosal inflammatory mediators (inflammasome) for control of offending bacteria which are downregulated after elimination of the offence so that host tissues are not damaged. IBD occurs when these regulatory pathways go awry predisposed by genetic abnormalities. Basic research regarding intestinal inflammation may reveal new insights into the role of the inflammasome, and the macromolecular complex of metabolites formed by intestinal bacteria (metagenomics) which will lead to the development of biomarkers that target specific pathogenic mechanisms of IBD. The advanced knowledge of antimicrobial protein expression in IBD can lead to its potential use as biomarkers for disease activity and also help in analysing the pathophysiology of IBD from their mechanism of protection against pathogens.

Biomarker also have great potential role in all phases of care of IBD patients. For those with suspected IBD, biomarkers can select patients unlikely to have IBD for whom further testing may be avoided. In diagnosed cases, biomarkers can be used to (1) determine the type of IBD and to predict disease course; (2) identify patients most likely to respond to

therapies and those that may require more aggressive therapies, thus determining the prognosis; and (3) differentiate patients with active inflammation from those likely to have symptoms from other causes in patients with recurrent symptoms.

As treatment strategies (medical and surgical) and prognostic outcomes become more and more disease-specific, refinement in disease classification becomes mandatory. The present approach based on clinical features, existing biomarkers, radiology, endoscopy and histopathology falls well short of the desired target. On the contrary, emerging diagnostic techniques in the field of gastrointestinal endoscopy, molecular pathology, genetics, epigenetics, metabolomics and proteomics is showing promising results. Novel advanced endoscopic imaging techniques (like magnification endoscopy, chromoendoscopy, narrow band imaging, confocal microendoscopy, etc.,) and biomarkers can shed new light for the differential diagnosis of IBD, better reflecting diverse disease behaviours based on specific pathogenic pathways.

Hence progress in basic research is most important in India and should focus on identifying the genetic links and pathogenetic mechanisms of Indian IBD patients. Already studies have shown that the common genetic mutations associated with CD in the west are absent in Indian patients, novel mutations are being discovered and it is likely that pathogenetic mechanisms and hence treatment approach will not be the same as in the west. This will have to be aided by progress in the fields of "OMICS" (genomics, metagenomics, cellomics, proteomics, metabolomics), endoscopy and molecular pathology. Also detailed community based cohort studies are needed to define the causal environmental factors and these should include the effects of (1) highly prevalent diabetes mellitus; (2) increasing obesity; and (3) repeated use of antibiotics and prolonged use proton pump inhibitors, all of which are known to alter the gut microbiota and can be involved in disease pathogenesis.

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Regulation of the serotonin transporter in the pathogenesis of irritable bowel syndrome

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Abstract

Serotonin (5-HT) and the serotonin transporter (SERT) have earned a tremendous amount of attention regarding the pathogenesis of irritable bowel syndrome (IBS). Considering that enteric 5-HT is responsible for the secretion, motility and perception of the bowel, the involvement of altered enteric 5-HT metabolism in the pathogenesis of IBS has been elucidated. Higher 5-HT availability is commonly associated with depressed SERT mRNA in patients with IBS compared with healthy controls. The expression difference of SERT between IBS patients and healthy controls might suggest that SERT plays an essential role in IBS pathogenesis, and SERT was expected to be a novel therapeutic target for IBS. Progress in this area has begun to illuminate the complex regulatory mechanisms of SERT in the etiology of IBS. In this article, current insights regarding the regulation of SERT in IBS are provided, including aspects of SERT gene polymorphisms, microRNAs, immunity and inflammation, gut microbiota, growth factors, among others. Potential SERT-directed therapies for IBS are also described. The potential regulators of SERT are of clinical importance and are important for better understanding IBS pathophysiology and therapeutic strategies.

Key words: Irritable bowel syndrome; Serotonin; Serotonin transporter; Regulation; Therapy

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Core tip: The serotonin transporter (SERT) participates in metabolizing serotonin in the gut and plays a crucial role in the pathogenesis of irritable bowel syndrome (IBS). This review summarizes the relevant evidence on the factors that might regulate SERT, including SERT gene polymorphisms, microRNAs, immunity and inflammation, gut microbiota and growth factors. This review also reveals several potential treatments targeting SERT for IBS patients.

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INTRODUCTION

As a functional bowel disorder, irritable bowel syndrome (IBS) has the highest incidence rate worldwide. IBS is defined as a disorder with complex symptoms appearing as abdominal pain/discomfort and altered bowel patterns^[1-3]. A growing number of people suffer from IBS, with an estimated 5.8%-17.5% prevalence, especially in females^[4,5]. IBS causes a tremendous decline in the health-related quality of life and brings a considerable socioeconomic burden of up to \$19 billion^[2,6]. The Rome III criteria have been improved to help with the diagnosis and differential diagnosis of the syndrome^[7-10]. According to these criteria, IBS can be divided into 4 subtypes, namely IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), IBS mixed type (IBS-M) and IBS unsubtyped (IBS-U)^[11,12]. Furthermore, a 6-year follow-up study showed that approximately 10% of patients with infective gastroenteritis suffer from post-infective IBS (PI-IBS)^[13]. Because IBS is considered to be a multifactorial and heterogeneous disease with various phenotypes, no single mechanism entirely explains the pathophysiology of the disorder. Some possible mechanisms involve the initiation, persistence and severity of symptom flares, including inflammation, immunity, infection^[14,15], the gut microbiota^[16,17], psychosocial stress^[16,18,19] and an abnormal brain-gut axis^[16,20]. Recent discoveries have revealed that genetic susceptibility^[21], diet/drug intolerances^[22] and environmental pollutants^[23] are closely associated with IBS pathogenesis. Although the etiology of IBS is largely elusive, there are some characteristic symptoms of the disorder, including visceral hypersensitivity^[16,24], intestinal barrier dysfunction^[25] and gut motility disorder^[16,17,26].

As a signal transducer and a neurotransmitter, serotonin (5-HT) mediates intercellular signaling transmission in the gut, and most of the 5-HT in the body is in the gut. Enteric 5-HT is synthesized

by enterochromaffin (EC) cells (90%) and enteric serotonergic neurons of the myenteric plexus (10%)^[27]. Therefore, EC cells are the main source of enteric 5-HT in the gastrointestinal (GI) tract^[28,29]. 5-HT inactivation is as important as 5-HT release for maintaining dynamic equilibrium. As a number of neurotransmitter sodium symporters or the solute carrier superfamily 6, the serotonin reuptake transporter (SERT) plays an irreplaceable role in 5-HT inactivation by removing 5-HT from the interstitial space in the lamina propria into mucosal enterocytes and presynaptic neurons that are responsible for catabolism^[30,31]. Coates *et al.*^[31] first characterized a significantly decreased level of SERT in IBS. However, there was another conflicting finding of increased SERT expression in IBS^[32,33]. Taking the significant differences in the analytical methodology used and the heterogeneity of phenotypes into account, most researchers, such as Faure *et al.*^[34], have demonstrated that IBS patients have a remarkably attenuated level of SERT expression in the intestinal lining, which conforms to a remarkably decreased capacity of enterocytes to reuptake 5-HT. It is generally accepted that there is a significant inverse correlation targeting the level of availability between SERT and 5-HT.

SERT plays a critical role in the uptake and internalization of extracellular 5-HT. Previous studies have provided support to the concept that SERT is regulated by transcriptional and posttranslational mechanisms. To date, an association between SERT gene polymorphisms and IBS susceptibility has been inconsistent among different ethnic groups and even among different populations^[35]. Despite the lack of consensus on the wide range of roles of potential factors, immunity activation, inflammatory response, gut microbiota and their relationships have been suggested to regulate SERT expression in PI-IBS^[36]. Probiotics are also notable for linking inflammation-immune systems and gut microbiota in IBS patients^[37]. Recent studies have also shed light on the fascinating roles of microRNAs, growth factors and other factors in regulating SERT^[38].

ROLE OF SERT IN IBS

5-HT expands its regulatory functions outside the central nervous system as a neurotransmitter. In the gut, 5-HT is also a key signal transducer^[39,40]. Although the complex roles of 5-HT in the gut have not yet been clearly and completely elucidated, current studies have proven that 5-HT acts upon mucosal sensory transduction, responding to pressure and luminal stimuli derived from diet and bacteria^[41]. The release of 5-HT acting on a series of 5-HT receptors initiates secretory reflexes, peristaltic reflexes and, if pronounced, diarrhea, by stimulating intrinsic primary afferent neurons and myenteric interneurons^[41-43]. Furthermore, by stimulating extrinsic sensory nerves, 5-HT can also transmit the sensation of discomfort to

Table 1 Summary of potential regulators of the serotonin transporter in irritable bowel syndrome

Regulatory factors	Ref.	Publication year	Study type
SERT gene polymorphisms			
5-HTTLPR	Zhang <i>et al</i> ^[78]	2014	Meta-analysis
	Areeshi <i>et al</i> ^[35]	2013	Meta-analysis
	Wang <i>et al</i> ^[73]	2012	Case-control study
	Yeo <i>et al</i> ^[74]	2004	Case-control study
	Kumar <i>et al</i> ^[75]	2012	Case-control study
	Sikander <i>et al</i> ^[76]	2009	Case-control study
	Pata <i>et al</i> ^[77]	2002	Case-control study
STin2 VNTRs	Wang <i>et al</i> ^[79]	2004	Case-control study
	Yeo <i>et al</i> ^[74]	2004	Case-control study
SNPs	Kohen <i>et al</i> ^[58]	2009	Case-control study
MicroRNAs (↓)			
MiR-16	Baudry <i>et al</i> ^[38]	2010	Experimental study
MiR-545	Jensen <i>et al</i> ^[64]	2009	Experimental study
MiR-15a	Moya <i>et al</i> ^[62]	2013	Experimental study
MiR-24	Liao <i>et al</i> ^[96]	2016	Case-control study
Immunity and inflammation			
Immune cells (↓)			
IELs	Foley <i>et al</i> ^[52]	2011	Experimental study
	Faure <i>et al</i> ^[34]	2010	Experimental study
Mast cells	Foley <i>et al</i> ^[52]	2011	Experimental study
T cells	Wheatcroft <i>et al</i> ^[104]	2005	Experimental study
	Faure <i>et al</i> ^[34]	2010	Experimental study
Inflammatory cytokines			
IFN-γ and TNF-α (↓)	Foley <i>et al</i> ^[105]	2007	Experimental study
TGF-β1 (↑)	Nazir <i>et al</i> ^[107]	2015	Experimental study
Gut microbiota			
EPEC (↓)	Esmaili <i>et al</i> ^[118]	2009	Experimental study
EcN (↓)	Nzakizwanayo <i>et al</i> ^[119]	2015	Experimental study
LGG (↑)	Wang <i>et al</i> ^[121]	2015	Experimental study
Growth factors (↑)			
EGF	Kekuda <i>et al</i> ^[132]	1997	Experimental study
bFGF	Kubota <i>et al</i> ^[133]	2001	Experimental study
NGF	Gil <i>et al</i> ^[134]	2003	Experimental study

5-HTTLPR: 5-HT-transporter-gene-linked polymorphic region; STin2 VNTRs: Variable number of tandem repeats STin2; SNPs: Single nucleotide polymorphisms; IELs: Intraepithelial lymphocytes; IFN-γ and TNF-α: Interferon-γ and tumor necrosis factor-α; TGF-β1: Transforming growth factor-β1; EPEC: Enteropathogenic *E. coli*; EcN: *Escherichia coli* Nissle 1917; LGG: *Lactobacillus rhamnosus* GG supernatant; EGF: Epidermal growth factor; bFGF: Basic fibroblast growth factor; NGF: Nerve growth factor.

the central nervous system along the gut-brain axis in IBS. Therefore, 5-HT is closely related to secretion, motility and sensation in the gut^[28,31]. Shufflebotham *et al*^[44] highlighted the importance of 5-HT dysfunction in IBS symptoms and psychophysiological manifestation with the use of the acute tryptophan depletion paradigm. Moreover, increasing evidence suggests that psychiatric comorbidities are highly prevalent in IBS patients^[45]. Antidepressant selective serotonin reuptake inhibitors (SSRIs) are considered to be possible treatments for IBS. In 2014, a systematic review declared that antidepressants are effective in treating IBS^[46]. However, in 2015, a meta-analysis with conflicting results found that the efficacy of SSRIs

to treat IBS was inconclusive^[47]. One study showed that IBS patients with a psychiatric comorbidity had a greater probability of carrying SERT variants^[48]. The possibilities underpinning antidepressants, such as SSRIs and other factors that regulate SERT, require further elaboration.

Termination of the 5-HT signal is as important as its initiation; therefore, SERTs on the cell membrane of enterocytes are vital to transport 5-HT intracellularly, where 5-HT is metabolized by monoamine-oxidases^[49]. Using mice with a targeted deletion of SERT, Chen *et al*^[50] demonstrated that nearly all of the intestinal epithelial cells on the surface of the lumen express SERT. As a result, it is not surprising that the intestinal mucosa has a huge capacity for taking up 5-HT from the interstitial space. Therefore, 5-HT is transported into enterocytes by SERT after release from EC cells and acting on local selected receptors^[30]. As a membrane-embedded transporter, SERT is crucial for modulating the amplitude and duration of the 5-HT signal^[51]. As discussed previously, a significant correlation has been observed between abnormalities of 5-HT signaling and IBS-like pathogenesis. Furthermore, it is now believed that altered SERT expression is responsible for disorganized 5-HT signaling. When dysregulated SERT increases mucosal 5-HT availability, high-levels of gut secretion and motility might accelerate the development of IBS-D^[52]. It is generally accepted that the abnormalities of SERT expression contribute to IBS development. However, the regulation of SERT expression in IBS and the underlying mechanisms are not fully understood.

POTENTIAL REGULATORY FACTORS OF SERT

Both genetic and non-genetic factors are implicated in the up-regulation or down-regulation of SERT expression in IBS (Table 1). It is becoming clear that genetic predisposition might underlie IBS in individuals^[53]. A large-scale study between monozygotic twins and dizygotic twins proved that both heredity and the environment contribute to the development of IBS. Furthermore, it appeared that environmental influence was more important for individuals than heredity in IBS^[54]. In the present article, the potential regulatory factors of SERT expression are presented and discussed, and these factors might be involved in the pathophysiology and/or etiology of IBS.

SERT gene polymorphisms

As Hotoleanu *et al*^[55] demonstrated using twin studies, familial aggregation and epidemiology, genetic factors contribute to IBS, especially polymorphisms of the SERT gene. In other words, a low-expression SERT genotype might underlie a genetic predisposition to IBS^[56,57]. Furthermore, Kohen *et al*^[58] reported a trend

towards an association between 5-HT-transporter-gene-linked polymorphic region (5-HTTLPR) L/L genotype and IBS. However, Camilleri *et al.*^[59] found that colonic mucosal expression of the SERT gene was normal in IBS. Galligan *et al.*^[60] found increased serotonin availability in SERT knockout rats associated with visceral hypersensitivity. The *SERT* gene, solute carrier family 6 member 4 (SLC6A4), localizes to chromosome 17q11.2. SLC6A4 spans approximately 40 KB, contains 14 exons and ultimately encodes a 603-amino acid protein^[61-63]. There are a series of polymorphic regions that might affect the expression or function of SERT^[59,64-67] and further alter 5-HT reuptake, reaching up to 40-fold *in vitro*^[68]. Current research mainly focuses on positive associations of the SLC6A4 genetic polymorphisms with the etiology of IBS, including 5-HTTLPR^[69], a variable number of tandem repeats (VNTR) STin2^[65] and functional single nucleotide polymorphisms (SNPs; rs25531 and rs25532, *etc.*)^[58,70,71]. However, the presence of linkage disequilibrium between the three aspects has not yet been determined^[58].

The most frequently studied variant, a 5-HTTLPR insertion/deletion polymorphism of approximately 44 base pairs, is subdivided into long (L) and short (S) alleles^[69,72]. Furthermore, compared with the L/S and S/S genotypes, the transcriptional efficiency of the L/L genotype is significantly higher^[73]. Our previous study found that the L/L genotype leading to a higher SERT level appeared more frequently in IBS-C individuals than in IBS-D and healthy individuals^[73]. Yeo *et al.*^[74] reported that the 5-HTTLPR polymorphism was highly related to female patients with IBS. The S allele leading to decreased transcription of SLC6A4 and attenuated expression of SERT protein resulted in a reduced reuptake of 5-HT and a higher 5-HT level, which was consistent with manifestations of IBS-D compared with other subtypes of IBS and controls^[75]. Contradictorily, Sikander *et al.*^[76] and Pata *et al.*^[77] reported that the S/S genotype had a significant correlation with IBS-C patients in the Indian and Turkish population, and Wendelbo *et al.*^[33] concluded an increased content of SERT availability in ileal epithelia facilitating the pathogenesis of IBS, regardless of the subtype. However, because of insufficient patients participating in these studies, there was still no consistent conclusion. A meta-analysis containing thousands of IBS cases found ethnic differences in the relationship between 5-HTTLPR and IBS; moreover, the L/L genotype, or rather the L allele, was more relevant to IBS-C in East Asians than in Caucasians^[78]. Similarly, another meta-analysis showed that the SLC6A4 polymorphism is associated with a reduced risk of IBS in American and Asian populations^[35].

Another SERT gene polymorphism, called variable number of tandem repeats STin2, or simply "STin2 VNTR" for short, is located in intron 2 and consists of an indeterminate number of 17-bp segments (*i.e.*, 9, 10 or 12 repeats)^[65,70]. Our previous study reported

that the 10/12 genotype might contribute to IBS^[79], although other reports regarding the association between STin2 VNTRs and IBS were controversial and inconclusive^[74,80]. With regard to functional SNPs within the VNTR promoter, Kohen *et al.*^[58] found that compared with the more frequent A-allele, the comparatively rare rs25531 G-allele decreased SERT transcription and thus increased the IBS risk by approximately 3-fold. SERT gene promoter polymorphisms have been implicated in the treatment effects of histone deacetylase inhibitors (butyrate or trichostatin) in cultured colonic epithelial cells (Caco-2 cells), which resulted in reduced SERT mRNA and protein expression by suppressing the human SERT (hSERT) promoter 1^[81]. The development of SERT gene-specific therapeutics to regulate SERT expression in the treatment of multiple disorders, including IBS, is realizable. Clinicians could put individualized treatment into effect according to different SERT genotypes as one of the factors.

MicroRNAs

Posttranscriptional gene regulation by microRNAs (miRNAs) can greatly contribute to miRNA-targeted gene translation^[82,83]. miRNAs, endogenous about 22 nucleotide (nt) noncoding RNAs, pair with and then silence target mRNAs and achieve fine adjustments of protein outputs^[84-86]. Of interest, nearly all aspects of biological processes, including development and cellular homeostasis, are under the influence of miRNAs. Moreover, miRNAs can facilitate the development of several types of diseases when they dysregulate targeted gene expression^[83-85,87]. Despite insufficient studies focusing on the 3'-untranslated region (3'-UTR) of SLC6A4, miRNA binding to the 3'-UTR of SERT mRNAs by incomplete complementary base pairing is crucial for SERT mRNA translation, localization and stability^[38,88].

During the past several years, it has been shown that SERT is a target of microRNA-16 (miR-16). The highly conserved miR-16 among mammalian species has high expression levels in the heart, brain, small intestine, lung and kidney^[89,90]. Baudry *et al.*^[38] investigated if SERT expression was decreased by miRNAs in monoaminergic neurons utilizing the 1C11 neuroectodermal cell line expressing SERT transcripts. The results showed a 40% decline in the numbers of [³H]-paroxetine (SSRI) binding sites after transfection with a high level of miR-16. SSRI fluoxetine down-regulated SERT expression by increasing the level of miR-16 in 1C11^{5-HT} cells (1C11 neuroectodermal cells differentiate into serotonergic neuronal cells)^[38]. Similar findings were obtained in the hippocampus, showing that fluoxetine treatment resulted in down-regulated miR-16 and 5-fold increased SERT expression, with further illustration that the level of miR-16 was regulated by SSRI antidepressants and was increased or decreased according to the different regions in

the brain. Furthermore, the neutralization of miR-16 played an antidepressant role in the hippocampus^[91]. Direct injection of anti-miR-16 had an antidepressant effect similar to fluoxetine^[91,92]. A study investigating acute lung injury also drew the same conclusions that decreased miR-16 levels contributed to increased SERT expression and therefore promoted the pathogenesis of pulmonary edema^[93].

miR-16 might not be the only modulatory miRNA involved in the translational repression of SERT. For example, Jensen and colleagues^[94] found that SERT expression in the HeLa cell line was also regulated by miR-545, and a U to G SNP in the 3'-UTR of the SERT mRNA had no effect on miR-545 binding and SERT down-regulation. In addition, miR-15a contiguously located at chromosome 13q14.3 with miR-16 also regulated SERT expression in rat and human cells^[62,89]. More concerning, the observed results from the brain tissue of Wistar rat pups highlighted that *Cronobacter sakazakii* infection up-regulated miR-16 expression interacting with SERT mRNA, which led to decreased levels of 5-HT and SERT expression^[95]. Recently, a study directly illuminated that increased miR-24 expression in the enterocytes of IBS patients and mouse models promoted IBS-D pathogenesis by down-regulating SERT expression^[96]. Discovering novel miRNAs related to posttranscriptional SERT gene regulation and elucidating the underlying mechanisms provide a new strategy to expand our understanding of miRNAs in the development and treatment of IBS.

Immunity and inflammation

Given that accumulating evidence points to a critical role for immune activation of the gut mucosa in EC cell hyperplasia and reduced SERT activity in IBS-D patients or post-infectious IBS (PI-IBS) patients^[97], it is not surprising that mucosal 5-HT is increased in IBS-D patients^[41,52,98,99] and PI-IBS patients^[41,98,100,101]. It is generally accepted that there are increased levels of mucosal immune cell infiltration and proinflammatory cytokines in IBS patients. Furthermore, the inflammatory state of the intestinal mucosa promotes visceral hypersensitivity^[14,34,102]. Evidence suggests that 50% of IBS patients exhibit a drastic 72% increase of immunocytes in colonic mucosa, including CD3⁺, CD4⁺ and CD8⁺ T cells and mast cells, compared with healthy controls^[41,103]. Foley *et al.*^[52] found that the reduced level of mucosal SERT mRNA in IBS-D patients was correlated with increased numbers of mucosal intraepithelial lymphocytes (IELs) and mast cells compared with healthy controls. A study from Wheatcroft and colleagues^[104] evaluated post-*Trichinella spiralis* infection of T cell receptor (TCR) knockout mice with respect to EC cell numbers and SERT expression. The authors demonstrated that deficiencies of all T cells decreased infection-induced EC cell hyperplasia and extinguished mastocytosis, with a drastic reduction in jejunal SERT expression. Paradoxically, despite

the general presence of inflammatory infiltrates, Faure *et al.*^[34] detected no differences in the numbers of IELs and CD3⁺ cells located in the lamina propria between IBS patients and healthy controls.

Accumulating evidence has demonstrated that proinflammatory mediators, such as interferon- γ and tumor necrosis factor (TNF)- α , and not solely a non-specific change of inflammatory damage on epithelial cells, induce significant reductions in SERT mRNA, SERT protein levels and SERT function in Caco2 cells^[105]. However, prostaglandin E₂ and interleukin-12 (IL-12) had no effect on the SERT mRNA and protein levels^[105]. Furthermore, treatment with Shugan decoction, a type of traditional Chinese medicine used to treat IBS-D patients, resulted in a decreased TNF- α level with up-regulated SERT gene and protein levels in colonic tissue, which suggested underlying interactions between TNF- α and SERT expression^[106]. A protective cytokine, transforming growth factor- β 1, can activate SERT activity and inhibit intestinal inflammation *via* PI3K and syntaxin 3^[107]. These studies provide an overview of immune mechanisms involved in SERT regulation in a subset of IBS patients.

Gut microbiota

It is generally accepted that gut microbiota dysbiosis is responsible for intestinal ecology disturbances, which could be a significant catalyst in the development of functional bowel disorders^[108,109]. The current insight is that gut host-microbial interactions are important elements involved in the pathogenesis of IBS because of the convincing findings that predisposed individuals following infectious gastroenteritis suffer from PI-IBS and resemble patients with IBS-D^[110,111]. Because of the rapid evolution of analytical techniques, such as 16S rRNA-based microbiota analyses for profiling bacteria in the GI tract, not just in culture, it has been shown that mucosal and fecal gut microbial community composition differs between patients with IBS and healthy controls^[112]. Albeit with significant differences in methods, many studies have found that the relative abundances of the genera *Lactobacillus*, *Bifidobacterium*, *Actinobacteria* and *Bacteroidetes* were decreased, while *Proteobacteria*, *Firmicutes* and *Firmicutes: Bacteroidetes* ratios were increased in fecal samples of IBS-D patients^[110,113,114]. Malinen *et al.*^[115] even found an association between altered bacterial composition and subtypes of IBS, with a decreased amount of *Lactobacillus spp.* among IBS-D patients and an elevated amount of *Veillonella spp.* among IBS-C patients. However, the lack of large sample sizes and the heterogeneity of IBS symptoms represent limitations of these studies.

As noted previously, particular gut microbes and microbial metabolites regulate tryptophan metabolism, the serotonergic system and brain-gut axis functions and thereby alter the levels of 5-HT in the colon and blood, which might suggest a critical role for

the intestinal flora in regulating SERT and ultimately influencing the pathogenesis of IBS^[40,112,116,117]. Yano *et al.*^[116] found that EC cells were promoted to synthesize and secrete 5-HT by endogenous bacteria, such as spore-forming bacteria and their metabolites in germ-free mice. Esmaili *et al.*^[118] found that Caco-2 cells and mice infected by enteropathogenic *E. coli* to simulate infectious diarrheal diseases (PI-IBS and enteric infections) had decreased SERT mRNA levels, apical SERT activity, 5-HT uptake and mucosal 5-HT content. An investigation by Nzakizwanayo *et al.*^[119] demonstrated that the exposure of mouse ileal tissue to *E. coli* Nissle 1917 *in vitro* increased 5-HT bioavailability and decreased its metabolite level [5-hydroxy indole acetic acid (5-HIAA)], which suggested the underlying mechanisms for clearing 5-HT by SERT. Similarly, in IBS, reduced 5-HIAA levels and 5-HIAA/5-HT ratios elucidate serotonergic system dysbiosis with regard to both synthesis and metabolism^[120]. Our previous study suggested that the supernatant of probiotics, such as *Lactobacillus rhamnosus* GG, up-regulated the SERT mRNA level as much as 9.4-fold in enterocytes and mouse intestinal tissues in a concentration- and time-dependent manner^[121,122]. Our research also found that a protein derived from LGG, known as p40, activated epidermal growth factor receptor (EGFR), which suggested that LGG up-regulated SERT possibly by activating EGFR^[123].

Therapeutic strategies targeting the gut microbiota to recover the decreased diversity and stability might be a viable treatment strategy for IBS and other 5-HT-related brain-gut-microbiota axis disorders^[40,116]. To date, scientists and clinicians have made a variety of creative attempts, especially using probiotics, prebiotics, antibiotics and fecal microbiota transplantation (FMT), to increase the relative abundance of commensals (such as *Lactobacilli* and *Bifidobacteria*, *etc.*) and conversely, to decrease the relative abundance of those bacterial species exacerbating IBS symptoms (*Clostridium*, *E. coli*, *Salmonella*, *Shigella* and *Pseudomonas*)^[108,124]. Both a low-carbohydrate diet and the probiotic LGG have been proven effective in IBS patients^[122,125]. *Lactococcus lactis*, which is effective in suppressing colon inflammation by secreting IL-10, restores colonic 5-HT concentrations, given that the 5-HT level is increased in a dinitro-benzenesulfonic-acid micro-inflammation model^[102]. Similarly, Martín *et al.*^[126] found that the probiotic *Faecalibacterium prausnitzii* strain A2-165 (a type of commensal bacterium) or its supernatant had anti-inflammatory effects, with down-regulation of 5-HT levels to restore the normal state. Rifaximin, the most studied antibiotic in IBS, increased the relative abundance of *Lactobacillus* in the ileum, which relieved the mucosal inflammatory state and visceral hyperalgesia of the rat model^[127]. There is growing evidence regarding the efficacy of FMT in relieving symptoms in IBS patients, even in patients with longstanding refractory IBS-D, *via* restoring the intestinal microbiota^[128-131]. However, no study has

demonstrated a relationship between FMT and SERT in IBS. Further studies are necessary to determine new classes of probiotics and underlying mechanisms contributing to the treatment of IBS; meanwhile, the feasibility and reliability of FMT remain to be determined.

Growth factors

There is growing evidence regarding the role of growth factors, such as EGF^[132], basic fibroblast growth factor^[133] and nerve growth factor^[134], in the up-regulation of SERT expression. At present, EGF has been the most studied of these factors. As a polypeptide with 53 amino acid residues and growth hormone^[135], EGF plays multiple biological roles by combining with a specific EGFR located on the basolateral surface of enterocytes^[135-138]. There is evidence to suggest that EGF is involved in many normal physiological processes (stimulating intestinal epithelium cell proliferation, differentiation and maturation^[136,139-141], *etc.*) and pathophysiological situations (maintenance of homeostasis^[142], protection and regeneration of gastrointestinal mucosa^[136,140,143]). Given that EGF signaling protects the GI tract from intestinal inflammation^[137], little is known about a potential correlation between EGF signaling and IBS pathogenesis.

In response to SERT regulation, as Gill *et al.*^[144] first suggested, EGF acting on EGFR activates the hSERT promoter and upregulates SERT mRNA levels and function in enterocytes through transcriptional mechanisms in a dose- and time-dependent manner. Two types of alternate promoters of the *SERT* gene, hSERTp1 and hSERTp2^[145], are both active in Caco-2 cells by approximately 2- to 2.5-fold, respectively, compared with the transfected results of the pGL2 empty vector alone^[144]. Accumulating evidence suggests that EGF promotes SERT gene expression. Kekuda *et al.*^[132] found that the treatment of human placental choriocarcinoma cells with EGF increased the levels of SERT transcriptional activity, SERT mRNA expression and SERT function, likely by activating the EGF receptor through tyrosine phosphorylation. Kubota *et al.*^[133] reached similar conclusions about EGF and basic fibroblast growth factor using human glial cells (astrocytes). However, the positive effects of EGF on both distinct promoters of the *SERT* gene (hSERTp1 and hSERTp2) are counteracted by inhibiting EGFR tyrosine kinase activity^[132,144]. Decreased plasma and colonic tissue EGF levels were observed in IBS patients and in a rat model with visceral hypersensitivity^[146]. Therefore, decreased EGF correlates with decreased SERT activity, which is consistent with the conclusions that decreased EGF levels result in decreased removal of 5-HT into intestinal epithelial cells, stimulating visceral sensitivity and ultimately contributing to IBS^[146]. As a neuroendocrine mediator, neurotrophin nerve growth factor is increased in mucosal tissues^[147,148] and could relieve intestinal barrier dysfunction and visceral hypersensitivity of IBS-D patients^[149,150]. These findings

suggest that the up-regulation of SERT expression and function by growth factors might provide a better understanding of the pathogenesis and treatment of IBS.

Others

In addition, several different factors modulate SERT expression. As an agonist of tyrosine-kinase receptors, aurintricarboxylic acid plays a role in the upregulation of SERT, similar to EGF^[132]. Although studies have found that some factors (CCAAT/enhancer binding protein beta^[151], heterogeneous nuclear ribonucleoprotein K^[152], 10(-7)M 4-β-12-tetradecanoylphorbol-13-acetate^[153], etc.) regulate SERT, it remains to be determined if these factors are involved in IBS pathogenesis.

FUTURE PROSPECTS

It is now believed that 5-HT signaling is essential to the pathogenesis of IBS. As a result, new therapeutic strategies targeting the abnormal expression of SERT might represent a breakthrough to relieve the symptoms of this excruciating disease^[28,109]. At present, therapeutic approaches targeting gut microbiota, immune activation and the inflammatory response have received adequate attention to regulate SERT. There is no doubt that these potential regulators of SERT hold great promise for the development of treatments for IBS.

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Gastrointestinal disorders associated with migraine: A comprehensive review

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Abstract

Migraine is a recurrent and commonly disabling primary headache disorder that affects over 17% of women and 5%-8% of men. Migraine susceptibility is multifactorial with genetic, hormonal and environmental factors all playing an important role. The physiopathology of migraine is complex and still not fully understood. Many different neuropeptides, neurotransmitters and brain pathways have been implicated. In connection with the myriad mechanisms and pathways implicated in migraine, a variety of multisystemic comorbidities (*e.g.*, cardiovascular, psychiatric and other neurological conditions) have been found to be closely associated with migraine. Recent reports demonstrate an increased frequency of gastrointestinal (GI) disorders in patients with migraine compared with the general population. *Helicobacter pylori* infection, irritable bowel syndrome, gastroparesis, hepatobiliary disorders, celiac disease and alterations in the microbiota have been linked to the occurrence of migraine. Several mechanisms involving the gut-brain axis, such as a chronic inflammatory response with inflammatory and vasoactive mediators passing to the circulatory system, intestinal microbiota modulation of the enteric immunological milieu and dysfunction of the autonomic and enteric nervous system, have been postulated to explain these associations. However, the precise mechanisms and pathways related to the gut-brain axis in migraine need to be fully elucidated. In this review, we survey the available literature linking migraine with GI disorders. We discuss the possible physiopathological mechanisms, and clinical

implications as well as several future areas of interest for research.

Key words: Migraine; Headache; Microbiota; Review; Gastrointestinal diseases

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Core tip: Migraine is a recurrent and disabling primary headache disorder that commonly affects a significant proportion of the global population. Recent reports demonstrate an increased frequency of gastrointestinal (GI) disorders in patients with migraine compared with the general population. We review the available literature linking migraine with GI complications and comorbidities.

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INTRODUCTION

Migraine is a disabling primary headache disorder, defined by the International Classification of Headache Disorders as recurrent, moderate to severe headache attacks lasting 4-72 h with associated features including nausea and/or vomiting^[1] that affects over 17% of women and 5%-8% of men^[2,3]. Recent publications have suggested that its worldwide prevalence may surpass 20%, and that it consistently rates as one of the most disabling conditions^[4]. The subtype of chronic migraine affects up to 2%-5% of the population worldwide^[5,6].

Susceptibility to migraine is thought to be multifactorial, with genetic hormonal and environmental factors all playing an important role. Unbiased genome-wide association studies have identified 13 migraine-associated variants pointing at genes that cluster in pathways for glutamatergic neurotransmission, synaptic function, pain sensing, metalloproteinases and vasculature^[7]. The physiopathology of migraine is complex and the precise mechanisms and pathways involved remain to be fully elucidated. Many different neuropeptides, neurotransmitters and brain pathways have been implicated, but whether pain generates in central or peripheral structures is a matter of debate. A review of all of these mechanisms is beyond the scope of this article, and excellent recent papers by Nosedá *et al.*^[8] and Burstein *et al.*^[9] thoroughly summarize the current concepts of migraine physiopathology.

Briefly, sequential steps of neurogenic inflammation, peripheral trigeminovascular input, and central cortico-

trigeminal nuclei activation are thought to mediate migraine pathogenesis. Headache arises initially from an inflammatory response in the dura, mediated by vasoactive peptides, including calcitonin gene-related peptide (CGRP), substance P and neurokinin A. These are all released by trigeminal fibers and lead to activation of nociceptive perivascular sensory nerve terminals located in the meningeal vasculature^[8]. Perpetuation of headache is thought to be secondary to increased cortical responsiveness, while cortical spreading depression (CSD) has been hypothesized to represent the pathophysiological correlate not only for crisis onset but also for migraine aura.

The many stages of migraine headache from prodromal to postictal symptoms involve alterations in multiple cortical and subcortical structures. CSD itself can also activate the trigeminovascular system. Nociceptive input from trigeminal fibers converge onto the spinal trigeminal nucleus, which later modulates the activity of second-order structures such as the ventrolateral area of the upper cervical and medullary dorsal horn, the periaqueductal gray matter, rostral trigeminal spinal nuclei, brainstem reticular areas, superior salivatory nuclei and the cuneiform nuclei^[8,9]. Activity in these brain regions modulates the myriad of symptoms that follow migraine headache. The frequency of accompanying autonomic symptoms, such as nausea, vasoconstriction, vasodilation and diaphoresis as well as the participation of the hypothalamus, has led some investigators to propose that autonomic dysfunction may also play an important part in migraine pathophysiology.

It is widely known that migraine is associated with a variety of comorbidities, mainly cardiovascular, psychiatric and other neurological conditions. Hypertension, hyperlipidemia, sinusitis, asthma, pulmonary emphysema, insomnia, affective disorders and fibromyalgia have all been associated with migraine. Recent studies have found that gastrointestinal (GI) disorders also appear to be more frequent in patients with migraine (PWM) than in the general population^[10]. In this review, we survey the available literature linking migraine with GI complications and comorbidities.

HELICOBACTER PYLORI INFECTION

Interest in a possible association between *Helicobacter pylori* (*H. pylori*) infection (the most relevant cause of gastritis and peptic ulcer) and migraine first arose after this microorganism was recognized as the cause of myriad extra digestive manifestations, including neurological diseases^[11,12]. Its association with both cardiovascular (including typical functional vascular disorders such as primary Raynaud phenomenon) and autoimmune diseases has been established, possibly due to a chronic inflammatory response with local secretion to the circulatory system of numerous inflammatory and vasoactive mediators^[11,12]. Both vascular and inflammatory hypotheses have been proposed

as mechanisms mediating migraine physiopathology, making a link between *H. pylori* and migraine at least plausible enough to warrant investigation.

The evidence for a possible association between *H. pylori* infection and migraine is surrounded by some controversy. In one study^[13], investigators performed endoscopic procedures in 31 children with migraine and abdominal complaints and found a remarkably high prevalence of esophagitis (41.9%), corpus gastritis (51.6%), antral gastritis (38.7%) and duodenitis (87.1%). However, only 7 had *H. pylori*, thus failing to support an association between *H. pylori* and migraine.

A study assessing 200 subjects affected by primary headache found a *H. pylori* infection prevalence of 40%. It also found a higher prevalence of migraine without aura in infected patients^[14]. A similar study performed on 225 PWM found that 40% were colonized by *H. pylori*, and that the intensity, duration and frequency of migraine attacks were significantly reduced in all patients who underwent *H. pylori* eradication^[15]. These results were questioned by a later case-control study of 103 PWM and 103 control subjects, in whom the proportion of *H. pylori* infection was almost identical; in addition, there were no clinical or demographic differences between colonized and non-colonized migraine patients^[16]. In a follow-up of the former study^[17], 175 PWM were compared with 152 matched controls and investigators found no difference in prevalence of infection (40% vs 39%, respectively). However, among infected subjects, they found a significantly higher prevalence of CagA-positive strains in patients affected by migraine with aura compared with those affected by migraine without aura (41% vs 19%) and with controls (41% vs 17%), suggesting a pathogenic role for that specific strain of *H. pylori*. In contrast, a group of Turkish investigators compared the prevalence of *H. pylori* infection in 70 PWM and found a greater prevalence when compared to 60 matched controls, as well as a slight clinical benefit after eradication therapy^[18].

Besides different *H. pylori* strains, differences between migraine subtypes could also explain some of these inconsistent results. In a study on 49 PWM without aura, *H. pylori* infection was more prevalent compared to controls, and interestingly investigators also found much higher prevalence in PWM without family history or hormonal fluctuations, suggesting that *H. pylori* infection could be particularly relevant in patients with fewer known risk factors for migraine^[19]. However, an earlier study had reported that the association with *H. pylori* was exclusive for migraine with aura^[17]. In an attempt to resolve the controversy over the epidemiological association between migraine and *H. pylori*, a recent meta-analysis that included five studies and 903 patients found an overall *H. pylori* infection rate in PWM of 45%, compared to 33% in controls, with subgroup analysis finding greater infection rate of *H. pylori* in Asian patients compared to Europeans, but no difference among migraine

subtypes^[20]. Recently, a case-control study demonstrated higher IgM antibody titers against *H. pylori* in PWM compared to controls; also, they found a positive correlation between IgG antibody titers and severity of migraine^[21]. This suggests the importance of *H. pylori* active infection in migraine.

The previous epidemiological studies suggested that eradication therapy could be beneficial for migraine control, but in a non-controlled setting. In the only available double-blind, randomized, controlled clinical trial, 64 patients diagnosed with migraine-type headache were randomized to receive migraine treatment and *H. pylori* eradication treatment or migraine treatment and placebo^[22]. Using the Migraine Disability Assessment (MIDAS) questionnaire, on enrollment patients in the treatment group had more severe symptoms, but these differences disappeared after completing the study. Analysis of the change in MIDAS scores between baseline and completion of the study revealed a slight benefit for the treatment group.

As for possible pathophysiological mechanisms, few studies have made relevant findings. In small studies evaluating the redox state of PWM, *H. pylori* infection did not influence plasma accumulation of peroxidative substances, values of nitrite/nitrate concentrations or expression of systemic nitric oxide^[23,24]. Others have speculated that elevated levels of interleukin-10 might be implicated, considering that studies have shown elevations in this cytokine both in PWM as well as in patients with infection with *H. pylori*, particularly with CagA-positive strains^[25,26]. This has a therapeutic implication since the administration of sumatriptan (5-HT_{1D} receptor agonist) reduces the levels of interleukin-10 during a migraine attack^[26]. The serum levels of CGRP, which has been suggested as a biomarker for chronic migraine^[27], are also higher in those with *H. pylori*-associated duodenal ulcers compared with healthy controls^[28].

In conclusion, whereas epidemiological evidence appears to support an association between migraine and *H. pylori* infection, the data is limited and investigations should focus on subgroups of patients and of different ethnicities, and should consider the regional variations in *H. pylori* infection^[20]. Furthermore, intervention studies suggest a small benefit for eradication therapy, but the long-term benefit has not been established, and a possible intrinsic role for antibiotic or antacid treatment cannot be ruled out completely^[29]. Available studies are heterogeneous in both populations and treatments used, both in migraine control and *H. pylori* eradication.

IRRITABLE BOWEL SYNDROME

Irritable bowel syndrome (IBS), considered a neuro-gastroenterologic functional disorder, shares some environmental risk factors with migraine (predominately affecting the female sex and younger individuals), and could be associated with conditions of smooth muscle

dysfunction. IBS is associated with a number of extra-intestinal manifestations, and both diseases are widely prevalent and share many somatic and psychiatric comorbidities^[30]. In systematic reviews of the existing literature on IBS and its comorbidities, patients were found to present a 2-fold increase in other somatic disorders, suggesting a common physiopathological mechanism^[31]. Indeed, chronic headache was reported in 34%-50% of all IBS patients^[31,32].

Large population-based studies of the prevalence of IBS and accompanying symptoms show migraine, as well as heartburn, dyspepsia, flushing, palpitations and urinary symptoms, to be more common in patients with IBS^[33]. Specifically, out of 1620 subjects, 350 fulfilled criteria for IBS, and of these, 32% complained of migraine-type headache, compared with 18% in controls. Of course, within a population of PWM, comorbid constellations vary, differing in headache and psychosocial profiles, highlighting the heterogeneity of environmental and genetic factors^[34]. Nevertheless, an epidemiologic association has been since confirmed by various studies. In a cohort of 208 patients with IBS, 17% had migraine, compared to 8% in 1240 controls^[35]. A more recent cohort study investigating 97593 IBS patients exhibited a migraine prevalence of 6%, compared to 2.2% in healthy controls^[36]. A migraine cohort of 14117 newly-diagnosed patients presented similar results, with an adjusted incidence of IBS 1.95-fold higher than in controls (73.87 vs 30.14 per 10000 person-years), particularly in those under 30 years of age^[37].

There is some indication that comorbid headache disorders in IBS patients could negatively alter their clinical course. In a study of IBS patients comparing first-time attendees with chronic attendees to an outpatient clinic, 40% of first-time attendees complained of mild headache and 1% of severe headache, compared to 59% and 23% of the chronic attendees, respectively^[38].

In a prospectively identified, hospital-wide population of migraine patients, another study found that 70% of patients met the Rome III criteria for concurrent functional gastrointestinal disorders (FGID), with 40.4% meeting criteria for IBS^[39]. The authors also demonstrated a clear link between coexistent FGID symptoms, psychological comorbidity and worse scores in anxiety/depression scales.

In the search for a common pathogenetic mechanism for IBS and migraine, neuroendocrine factors, immunological factors, the brain-gut axis and even the intestinal microbiota have been postulated^[30]. A role for serotonin (5-HT) has been postulated in both diseases, as well as for other factors such as biopsychosocial dysfunction, heredity, genetic polymorphism, central/visceral hypersensitivity, somatic/cutaneous allodynia and the neurolimbic pain network^[30]. Another possible physiopathologic link could be derived from treatment studies. A recent small, clinical study, showed that an IgG-based food elimination diet could reduce

symptomatology and attack prevalence of both disorders in patients with comorbid IBS and migraine^[40].

GASTROPARESIS

Gastroparesis can be defined as delayed emptying of the stomach in the absence of mechanical obstruction, and its clinical manifestations include nausea, vomiting, bloating and weight loss, among other symptoms^[41]. An association between migraine and alterations in gastric motility has been noted, describing this motility disorder as a stomach with a functional "vagotomy"^[42]. A delay in gastric emptying time and increased pyloric tone characterized these alterations. The recognition of GI stasis in PWM led to initial concerns on the absorption of analgesics and the effect on therapeutic efficacy, but some authors emphasized the accentuation of overall distress and inconvenience caused by GI symptoms^[43]. Studies confirmed an effect of gastric emptying delay time over the absorption of paracetamol and acetylsalicylic acid, among other common analgesics^[44]. One study established the close association of gastroparesis with migraine attacks. PWM during a pain-free period showed normal gastric emptying times measured with an epigastric impedance method, but these times were significantly delayed during severe or moderate attacks, and delay times were significantly correlated with the intensity of headache, nausea and photophobia^[45].

The physiology of emesis during migraine attacks could, in some manner, mirror those of gastroparesis, and these considerations have had therapeutic implications, as both dopaminergic and 5-HT₄ agonists have prokinetic properties^[46]. Early findings of an apparently normal gastric motility outside of migraine attacks^[45] suggested that the physiopathology of gastroparesis in migraine was mediated by pain mechanisms, such as adrenergic and endogenous opiates, or to factors shared with the pathophysiology of a migraine attack itself. An excessive sympathetic response coupled with a decreased parasympathetic tone has also been postulated^[43]. However, a recent study with gastric scintigraphy showed that gastric emptying time is delayed in both ictal and interictal periods, suggesting an alteration in enteric autonomic function^[41,47]. Another study showed that PWM have meal-induced hypersensitivity of the stomach, due to a low postprandial discomfort threshold, irrespective of the presence of dyspepsia^[48].

More recent studies have further characterized migraine-associated gastroparesis, but these have also questioned the existence of an alteration outside the ictal period. Using gastric scintigraphy in 3 PWM, investigators found that gastric emptying delay occurs not only in ictal and interictal periods, but also in both drug-induced and spontaneous migraine attacks^[49]. However, other groups using similar methods were

not able to find differences in emptying time between patients without migraine and PWM in the interictal period^[50]. Of note, both studies included a very small sample, and only the latter included an age- and sex-matched control group. In a larger gastric scintigraphy study, involving 27 PWM and 12 healthy controls, again there was no gastric emptying delay interictally^[51]. However, in this study, PWM who experienced other GI symptoms were excluded. The controversy over this issue also stems from methodological issues, such as the criteria used to define gastric emptying delay, which should be adapted to the specific population studied, as well as small sample sizes and inadequate control group selection^[52].

Although there is enough evidence to link gastroparesis with migraine, the nature, causes, correlates and consequences of gastric stasis in migraine are just beginning to be elucidated^[41,53]. There is controversy over whether gastroparesis occurs both ICTALLY and interictally, but it is clear that it is associated with increased discomfort and affects the effectiveness and absorption of orally administered drugs. This suggests that non-oral formulations of commonly used migraine medications as well as the addition of prokinetic drugs could theoretically offer an advantage^[41]. At least one small trial showed an additive effect of a prokinetic drug (trimebutine) over the efficacy of rizatriptan, a 5-HT_{1B/1D} agonist^[54].

OTHER FUNCTIONAL GI DISORDERS

Other functional GI disorders have been linked to primary headaches, but there is doubt as to a specific link with migraine. In population-wide registries, both diarrhea and constipation are significantly more frequent in headache sufferers compared to the general population, with no apparent difference between PWM and non-PWM^[10]. In a cross-sectional study of 326 children, nearly 20% of those who complained of headaches had constipation, a significantly higher number than in controls^[55]. However, no such association was found among PWM. Another retrospective study of 96 patients with primary headache also found comorbid constipation in 25%, but this was mostly associated with tension-type headaches^[56]. All studies reported a close correlation of constipation with headache severity, suggesting that it is this factor, together with the related affective and emotional distress, that more adequately explains the association. In a well-designed web-based survey aiming to screen for symptoms of reflux, among 1832 PWM, 22% reported having the diagnosis of GERD, 11.6% reported experiencing heartburn, and 15.8% reported undiagnosed reflux symptoms^[57]. The most common used medications were triptans but a significant number used NSAIDS. Whether reflux symptoms are a side effect of these medications or are independently associated with migraine is unknown.

HEPATO-BILIARY DISORDERS

There is little evidence linking migraine with hepato-biliary disorders. In a community study on the prevalence of chronic complaints, migraine and some types of biliary colic or right upper abdominal quadrant discomfort occurred together with some regularity, but a statistical association was not established^[58]. In another study, 488 healthy patients and 99 migraine patients reported upper abdominal symptoms, including unexplained right upper quadrant discomfort. These symptoms were more than twice as frequent in PWM, after adjusting for age, sex, smoking and consumption of analgesics and alcohol^[59]. A study of twin samples including a cohort of 1200 patients, found an association between migraine and biliary tract disorders, with higher ORs in monozygotic twins (OR = 3.5) than in dizygotic twins (OR = 1.7-2.7)^[60]. Waist circumference and female sex were also associated with migraine, but the association with biliary tract disorders remained even after controlling for these factors. The stronger association in monozygotic twins suggests a genetic influence. A weakness of the study was that the migraine diagnosis was based on iterated questionnaires and personal interviews and not guideline-based criteria.

As with other functional GI disorders, such as IBS, functional biliary tract disorders would be expected to be more robustly represented in PWM. In a recent study involving 972 patients with biliary dyskinesia (over 80% women), 14.6% were PWM^[61], a proportion similar to worldwide prevalence^[4,62]. In this study, 30% of the cohort presented comorbid anxiety or depression as well. Interestingly, migraine was an independent predictor (OR = 2.13) for continued medical resource utilization (for recurrent attacks of biliary symptoms), which could suggest a more severe course in PWM.

In experimental studies, cholecystokinin (CCK) coexists with CGRP in the trigeminal ganglion; and trigeminal ganglion stimulation was able to induce local increases of CCK^[63]. CGRP is also able to influence biliary motility *in vitro*, and impaired CGRP release is associated to biliary tract disease in humans^[64,65]. This evidence is proposed as a possible common physiopathologic mechanism linking biliary tract disorders and migraine, in addition to a possible role of obesity and female hormones and a vasodilatory effect of CCK^[60,66].

An association between liver disease and migraine and other headache types is even rarer and of dubious physiopathological standing^[67]. A recent cross-sectional study showed that migraine patients with non-alcoholic fatty liver disease (NAFLD) had significantly more attacks and a higher frequency of auras, but also higher waist circumferences and metabolic disturbances^[68]. The study was not designed to establish an independent association of NAFLD and migraine, but did suggest a more aggressive disease with a simultaneous diagnosis. Obesity and metabolic

disturbances, which are important determinants of NAFLD, are also associated with an increased risk of migraine^[69,70].

Based on the available data, CCK, which seems to have a role in the physiopathology mechanism in migraine^[60,63,66], is released in response to fatty acids in the proximal intestine^[71,72]. In this respect, a cross-over study including 83 PWM randomly assigned to a low lipid diet or a normal lipid diet found that the number (2.9 ± 3.7 vs 6.8 ± 7.5 , $P < 0.001$) and severity [1 pt indicated mild, 2 pts moderate and 3 pts very severe headache (1.2 ± 0.9 vs 1.7 ± 0.9 , $P < 0.01$)] of migraine attacks significantly decreased during the low lipid diet intervention periods^[73]. Likewise, a cross-sectional study showed that after a weight loss program, those who achieved a significant weight loss and metabolic control presented improvement of migraine^[70].

CELIAC DISEASE

Celiac disease (CD) is an autoimmune condition that occurs in those genetically predisposed to dietary gluten hypersensitivity, affecting about 1% of the general population^[74]. Besides GI symptoms, it is now known to have systemic involvement. Neurological complications are well-known manifestations of CD, including epilepsy, occipital calcification and migraine-like headaches; also, celiac antibodies have been described in patients with a wide range of neurological disorders, including encephalopathy, ataxia, neuropathy and myopathy, among others^[75]. Nonetheless, it remains unclear whether gluten sensitivity contributes to the pathogenesis of these disorders or whether it represents an epiphenomenon. Moreover, case reports of patients with concomitant CD and migraine describe the total disappearance of headaches after treatment of CD, and others describe particular combinations of signs and symptoms, such as CD, cerebral calcifications and migraine^[76,77], suggesting the existence of a pathophysiological association. Other single-case reports have described migraine as the presenting symptom in patients later confirmed to have CD^[78].

The search for antibodies associated with CD in migraine patients aims at establishing an epidemiological and possibly a physiopathological association. In an early study on the association between celiac antibodies and children with neurological disorders, including migraine, epilepsy and hypotonia, among others, investigators found only 13% of cases of anti-gliadin IgG antibodies and no cases of endomysial antibodies^[79]. Cases were not followed up with biopsies, but none seemed to have clinically evident CD. Another study involving 25 PWM (ages: 14–37; 22 females) did not find a single case of anti-gliadin antibodies in peripheral blood samples^[80]. However, the limitation of anti-gliadin antibodies is its low diagnostic accuracy; these antibodies have a high prevalence in normal population^[81]. Therefore, the reported association

between CD and migraine using this test may reflect a sampling error. In a more recent study of 100 children with migraine, only 2% had serologic tests positive for CD antibodies, a finding not different from a control group of 1500 healthy subjects^[82]. The only positive study, which included 73 children with migraine, found that 5.5% of patients have positive celiac antibodies compared with 0.6% in the control group^[83]. However, most of the seropositive children had normal duodenal biopsies, putting in doubt the diagnosis of CD. On the other hand, only 1 patient out of 147 controls was seropositive, a prevalence lower than what would be expected in the general population. Together, this data does not support screening for CD antibodies in PWM, but does not exclude a causal link in those affected with both disorders simultaneously.

The occurrence of few cases of CD in PWM and the high frequency of headaches in CD provide some support for a possible asymmetrical association. In a study of 72 adult patients with biopsy-proven CD who were screened for neurological disorders, 28% had migraine, among other neurological symptoms^[84]. A recent study of 188 patients with proven CD found that they had a significantly higher prevalence of migraine headaches compared with controls (OR = 3.79), particularly in women and those under 65 years of age^[85]. Additionally, there was a trend of more severe headaches in CD patients compared with controls. In a retrospective study of 354 children with CD, 24.8% had experienced headaches, compared to 8% in an age and sex-matched control group^[86]. The screening for migraine in patients with CD would, therefore, seem to be justified.

The occurrence of more severe headaches in patients with CD and migraine raises the question of a possible therapeutic effect of a gluten-free diet. In a study of 4 PWM and serologically and endoscopically confirmed CD, a gluten-free diet resulted in complete remission of migraine symptoms in one patient and improvement in frequency, duration, and intensity of migraine in the other 3^[87]. These improvements were associated with a reduction in single photon emission CT regional baseline brain tracer uptake in all 4 patients. Of note, these 4 patients were screened out of a population of 90 PWM (less than 5%). In another study of a cohort of Italian patients with CD, migraine-type headaches were more common than in controls (32%) and subsided partially with gluten-free diet^[88]. Other large cohorts of CD patients have similarly described improvement in migraine-type headaches with dietary intervention^[86,89].

Although the link between CD and migraine is not fully elucidated, they do share many psychosocial and pathophysiological characteristics. A physiopathological link is suggested by imaging studies. In a case series of 10 patients with CD and episodic headaches, brain MRI showed diffuse and heterogeneous hyperintensities involving white matter, similar to lesions described in PWM^[90]. The majority of those patients had symptom

Table 1 Summary of gastrointestinal disorders associated with migraine

GI disorder	Association	Proposed implicated mechanism	Clinical implication
<i>H. pylori</i> infection	Infection rate of <i>H. pylori</i> : 45% in PWM <i>vs</i> 33% in controls ^[20]	Chronic inflammatory response with inflammatory and vasoactive mediators passing to the circulatory system	Screening of <i>H. pylori</i> infection in patient with migraine
	Main affected: CagA-positive strains ^[17] Asian > Europeans ^[20]	↑ Interleukin-10 (CagA-positive strains) ^[25] ↑ CGRP ^[28]	Improvement of migraine with <i>H. pylori</i> eradication ^[17,18,22]
Irritable bowel syndrome	6%-32% migraine-type headache in IBS patients <i>vs</i> 2.2%-18% in controls ^[33,35,36]	The brain-gut axis and the intestinal microbiota have been postulated ^[30,95] Serotonin, biopsychosocial dysfunction, heredity, genetic polymorphism, central/visceral hypersensitivity, somatic/cutaneous allodynia, neurolimbic pain network ^[30]	Improvement of migraine with elimination diet ^[40]
Gastroparesis	During a migraine attack gastric emptying delay and impairment of drug absorption has been demonstrated ^[44,45]	↑ Sympathetic response ^[43] ↓ Parasympathetic tone ^[43] Dysfunction of enteric autonomic system ^[41,47]	Increase absorption of antimigraine agents by administering antidopaminergic and 5-HT4 agonists with antiemetic/prokinetic properties ^[46]
Hepato-biliary disorders	Association between migraine and biliary tract disorders ^[60] Genetic influence: In monozygotic pairs (OR = 3.5) In dizygotic pairs (OR = 1.7-2.7). Among the migraine characteristics, in those PWM with NAFLD, the presence of aura was higher (73.6% <i>vs</i> 26.5%), and the disease (9 yr <i>vs</i> 6 yr) and attack (72 h <i>vs</i> 48 h) durations were longer than in those without NAFLD ^[68] . Obesity and metabolic disturbances which are important determinants of NAFLD are also associated with an increased risk of migraine ^[69,70]	CCK has been found to coexist with CGRP in the trigeminal ganglion ^[63] . When stimulated induce local increase of CCK which has a vasodilatory effect ^[63,66] . CGRP has shown to influence biliary motility. The impaired CGRP release has been associated to biliary tract disease in humans ^[65]	Low-fat diet improves frequency and severity of migraine ^[73] In connection with NAFLD: Weight loss and metabolic control have shown to improve migraine ^[70]
Celiac disease	28% prevalence of migraine in subject with biopsy-proven CD ^[84] Higher prevalence of migraine in biopsy-proven CD group than in controls (21% <i>vs</i> 6%, OR = 3.79) ^[85] Main affected: Female Age < 65	Neurological complications in CD may be caused by a general inflammatory response ^[92] Elevated levels of interferon-gamma and TNF-alpha (both independently implicated in migraine and CD) modulate neuropeptide CGRP ^[93]	The screening for migraine in patients with CD seems to be justified. Possible therapeutic effect with gluten-free diet ^[86-89]

GI: Gastrointestinal; PWM: Patients with migraine; CGRP: Calcitonin gene-related peptide; IBS: Irritable bowel syndrome; CCK: Cholecystokinin; NAFLD: Non-alcoholic fatty liver disease; CD: Celiac disease; TNF: Tumor necrosis factor.

improvement with a gluten-free diet. Authors have speculated that a state of hypervigilance, associated with an exaggerated response to future threats and episodic attacks, could transform a genetically sensitive nervous system into one susceptible to the alterations underlying these chronic and disabling diseases^[91]. Another hypothesis is that neurological complications in CD may be caused by a general inflammatory response, rather than be directly antibody-mediated. This idea is supported by a study that showed no correlation between neural antigens and neurologic symptoms in patients with CD^[92]. Hypothetically, elevated levels of interferon-gamma and tumor necrosis factor-alpha, both independently implicated in migraine and CD, and known to modulate the neuropeptide CGRP, could explain the apparition/progression of migraine symptoms in patients with CD^[93].

A summary of GI disorders associated with migraine,

proposed physiopathological mechanisms, and clinical implications is presented (Table 1).

MIGRAINE AND THE GUT MICROBIOME

There is evidence that suggests gut microbiota can modulate the brain-gut axis through many pathways, with a potential to influence brain function and nociceptive behavior^[94,95]. The intestinal surface contains 100 trillion microorganisms, separated from the host by a layer of columnar intestinal epithelial cells. Indirect links have been made between gut microbiota and the function of the major pathophysiological mechanisms associated with migraine: Serotonergic transmission, CGRP activity and cortical reactivity^[95]. Dysbiosis has an impact on immune function, epithelial barrier permeability, absorption and metabolism of nutrients affecting, in consequence, GI and central

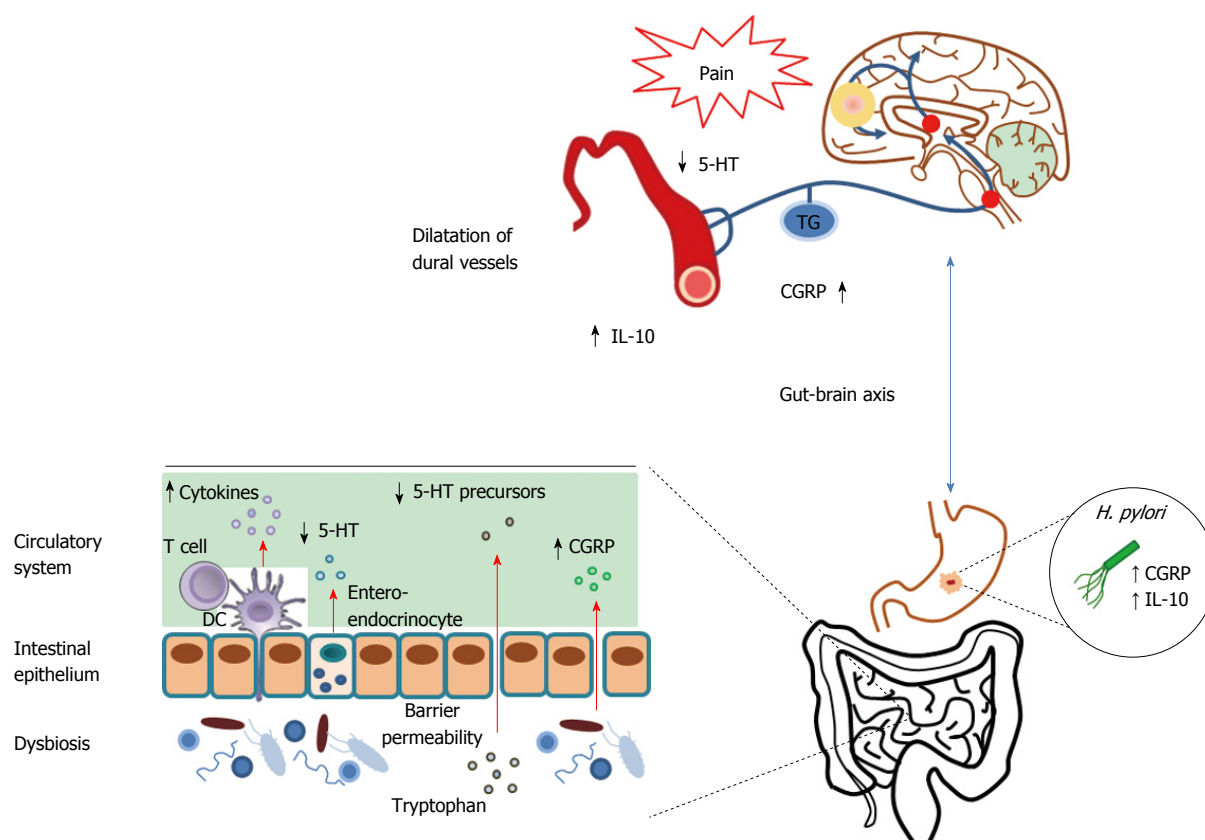


Figure 1 Role of the gut microbiota in migraine. Immunological, endocrine, metabolic and neural signals are important pathways by which the gut microbiota influences brain functions. Altered gut microbiota (dysbiosis) affects the normal assimilation of nutrients (tryptophan metabolism), barrier permeability, mucosal immune and enteroendocrine cells, affecting in turn some communication pathways; this results in the production of gut peptides (\uparrow CGRP) by certain microbes, abnormal release of cytokines (\uparrow IL-10) and hormones (\downarrow 5-HT). *H. pylori* also plays a role in the release of cytokines (IL-10) and CGRP. The increased cytokines and CGRP levels, as well as the decreased 5-HT levels, modulate the vasodilatory response of dural vessels, triggering and perpetuating migraine attacks. DC: Dendritic cell; 5-HT: 5-hydroxytryptamine; IL: Interleukin; CGRP: Calcitonin gene-related peptide; TG: Trigeminal ganglion; *H. pylori*: *Helicobacter pylori*.

nervous system (Figure 1).

The serotonergic system has been shown to be differentially affected by the gut microbiota in experimental studies. Whole-brain tryptophan, tyrosine and glutamine levels are lower in germ-free mice compared to those re-colonized by normal microbiota^[96], while concentrations of 5-HT and 5-hydroxyindoleacetic acid in hippocampal slices are elevated in germ-free mice^[97]. Furthermore, circulating levels of 5-HT and tyrosine are elevated in germ-free animals, compared to those with normal gut microbiota^[97,98].

CGRP functions not only as a transmitter but also as a gut hormone, and its signaling could be influenced by microbiota through multiple pathways^[99]. Although studies demonstrate changes in the expression of sensory-related peptides in the gut by modulating the whole microbiome, no direct effect has been found for CGRP^[100]. However, at least one study did show that after treatment with the probiotic *Pediococcus acidilactici*, the number of CGRP-immunoreactive neurons increased in the submucosal plexus ganglia of the small intestine^[101], although no such effect was observed with *Saccharomyces boulardii*^[102].

Whether the gut microbiome has any effects on large-scale cortical function is a matter of theoretical

debate. In an interesting study, a group of healthy volunteers who were given fermented milk product with probiotics (*Bifidobacterium animalis* subsp *Lactis*, *Streptococcus thermophiles*, *Lactobacillus bulgaricus*, and *Lactococcus lactis* subsp *Lactis*) showed a reduced task-related response of a distributed functional network in affective and viscerosensory cortices on functional MRI^[103]. The results of a small, non-randomized trial, including 29 migraine patients, showed a significant reduction in migraine severity after 12 wk of probiotic supplementation compared to baseline^[104]. Moreover, an open-label study using a combination of two nutritional formulations (combination A: Enzymatically rendered fish protein high in bioactive peptides and amino acids plus probiotics and chlorophyll; combination B: Composed of 21 different ingredients designed to improve the nutritional status of the kidneys and liver) demonstrated a significant and sustained improvement in quality of life (determined by the Migraine Specific Quality of Life Questionnaire), supporting the idea that dysbiosis and altered assimilation of nutrients could have an important role in the pathophysiology of migraine^[105].

There is no direct evidence to conclusively support that the gut microbiome can affect migraine. However,

Table 2 Future areas of interest on gastrointestinal disorders associated with migraine**Unanswered questions and future directions***H. pylori*

- Ethnicity difference between *H. pylori* and migraine association
- Effects of different eradication therapy schemes in migraine
- Impact of routine screening for *H. pylori* infection in PWM
- Intrinsic role for antibiotic or antacid treatment used for *H. pylori* eradication in migraine
- Effect of triptans (5-HT_{1B} and 5-HT_{1D} receptor agonist) in PWM depending their *H. pylori* infection status

Irritable bowel syndrome

- Role of gluten-, wheat- and FODMAP-free diets in migraine
- Effect of “dysbiosis” over serum level of cytokines and neurotransmitters in migraine

Gastroparesis

- Nature, causes and consequences of gastroparesis in migraine
- Determination of gastroparesis occurrence during the ictally and interictally periods in migraine

Hepato-biliary disorders

- Prevalence of hepato-biliary disorders in different populations
- Role of CCKB (CCK-2) receptor antagonists in migraine
- Role of CCKA (CCK-1) receptors agonist in the treatment of obesity and migraine
- Effect of coffee consumption in migraine in patients with NAFLD

Celiac disease

- Routine screening for migraine in patients with CD
- Role of interferon-gamma and TNF-alpha in the apparition/progression of migraine in patients with CD

Microbiome

- Effects of normal microbiota and dysbiosis in CRGP regulation and expression
- Effects of normal microbiota and dysbiosis in the serotonergic system and migraine
- Role of fecal microbiota transplantation in migraine

Other GI disorders

- Reflux symptoms in patients with migraine as cause of the disease itself or a side effect of antimigraine medications

PWM: Patients with migraine; CCK: Cholecystokinin; NAFLD: Non-alcoholic fatty liver disease; CD: Celiac disease; TNF: Tumor necrosis factor; CRGP: Calcitonin gene-related peptide; GI: Gastrointestinal; *H. pylori*: *Helicobacter pylori*.

the prospects of a therapeutic strategy based on probiotic dietary interventions or modifications of the gut microbiome, considering that these would intuitively have a high safety profile and cost-effectiveness, make this issue an interesting topic for further research.

FUTURE AREAS OF RESEARCH

Several unanswered questions arise related to this topic. Therefore, further research in GI disorder associated to migraine is warranted in order to evaluate the real impact of some screening and therapeutic measures as well as to clearly define the common inflammatory and neurotransmitter pathways in GI disorders and migraine (Table 2).

CONCLUSION

Currently, sufficient evidence exists linking the increased frequency of several GI disorders with migraine compared to the general population. The gut-brain axis plays an important role in the association between GI disorders and migraine. Multiple inflammatory and vasoactive mediators are significantly implicated in the physiopathology of migraine, mainly through the GI microbiota modulation of the GI immunological and autonomic system.

Based on the several implicated mechanisms between different GI disorders and migraine, several pharmacologic and non-pharmacologic therapeutic

options for specific GI disorders have shown to improve frequency and severity of migraine attacks. Also, based on the implicated mechanisms, some screening measures (e.g., *H. pylori* infection) seem to be justified in PWM. Treatment of GI comorbidities in migraine might not only lead to a better quality of life but could also open roads for novel therapeutic strategies for this prevalent and disabling disease.

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Abstract

A growing body of epidemiologic research has demonstrated that metabolic derangement exists in patients with hepatitis B virus (HBV) infection, indicating that there are clinical associations between HBV infection and host metabolism. In order to understand the complex interplay between HBV and hepatic metabolism in greater depth, we systematically reviewed these alterations in different metabolic signaling pathways due to HBV infection. HBV infection interfered with most aspects of hepatic metabolic responses, including glucose, lipid, nucleic acid, bile acid and vitamin metabolism. Glucose and lipid metabolism is a particular focus due to the significant promotion of gluconeogenesis, glucose aerobic oxidation, the pentose phosphate pathway, fatty acid synthesis or oxidation, phospholipid and cholesterol biosynthesis affected by HBV. These altered metabolic pathways are involved in the pathological process of not only hepatitis B, but also metabolic disorders, increasing the occurrence of complications, such as hepatocellular carcinoma and liver steatosis. Thus, a clearer understanding of the hepatic metabolic pathways affected by HBV and its pathogenesis is necessary to develop more novel therapeutic strategies targeting viral eradication.

Key words: Hepatitis B virus infection; Nucleic acid metabolism; Metabolic derangement; Metabolic signaling pathway; Glucose metabolism; Lipid metabolism; Bile acid metabolism; Vitamin metabolism

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Core tip: Currently, hepatitis B virus (HBV) infection still poses a serious threat to public health, and causes

approximately 1 million deaths annually due to HBV-related liver diseases. Thus, investigation into the complex host cellular responses to HBV infection is a crucial area of research. Multiple epidemiologic data have proved that patients with HBV infection often have metabolic disorders. Therefore, we systematically reviewed the alterations in metabolic response to HBV infection with regard to molecular mechanisms. Deciphering the detailed interplay mechanisms would contribute to our understanding of HBV-induced pathological processes and may lead to nutritional therapies as new anti-HBV treatments.

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INTRODUCTION

Chronic hepatitis B (CHB) is a serious global health concern, which is estimated to affect approximately 350 million people worldwide and carries a significantly increased risk of serious liver disorders including liver cirrhosis, hepatic decompensation or hepatocellular carcinoma (HCC). Approximately, one million deaths occur each year due to CHB and its complications^[1,2]. However, the intrinsic mechanisms of hepatitis B virus (HBV)-induced diseases are unclear, and no complete cure is currently available for CHB. Thus, an investigation of the complex host responses to HBV infection is a crucial area of research, which in turn could provide a more thorough understanding of the pathogenesis and potential novel targets in antiviral drug discovery.

The HBV genome (3.2 kb) is a partially double-stranded, relaxed circular DNA, mainly controlled through the transcriptional activation of four promoters (core, X, pre-S1 and pre-S2/S) and two enhancers (EnhI and EnhII)^[3-6]. The recruitment of cellular transcription factors to the binding sites of HBV genome could regulate virus transcription. Some of these transcription factors are ubiquitous nuclear receptors such as C-AMP-response element binding protein, specificity protein 1, prospero-related homeobox protein and nuclear respiratory factor 1; some are liver-enriched nuclear receptors such as hepatocyte nuclear factor 4, alpha (HNF4a), CAAT enhancer-binding protein, peroxisome proliferator-activated receptors, alpha/retinoid X receptors, alpha (PPARα/RXRα) and farnesoid X receptor (FXR)^[7,8].

Interestingly, the native role of most HBV-bound transcription factors is the coordination and control of hepatic metabolism^[8]. For example, HNF4a plays a key role in glucose metabolism in the liver^[9]. PPARα

controls fatty acid β-oxidation and is a crucial regulator of genes involved in the cellular fasting response^[10]. FXR, activated by bile acids, is a molecular link between lipid metabolism and bile acid^[11]. Thus, it indicates that HBV has adopted a smart mode of regulation, which is similar to that of major hepatic metabolic genes, implying that there is an association between metabolism and HBV infection. Our previous work also suggested that activation of fatty acid oxidation-associated PPARα was required for fasting-induced HBV transcription^[12].

Accumulating epidemiologic evidence has shown that there is still debate regarding the clinical associations between HBV infection and host metabolism. For instance, patients with chronic HBV infection, compared with healthy adults, have lower triglyceride (TG) and high-density lipoprotein (HDL) levels, but a higher adiponectin level^[13]. A review by Janicko *et al.*^[14] described strong correlations between CHB and the metabolic syndrome, non-alcoholic fatty liver disease or dyslipidemia, whereas an inconclusive association between diabetes mellitus and CHB has also been described. However, in metabolic signaling pathways, an increasing number of studies have shown that HBV modulates all aspects of host hepatic metabolism. In order to understand the unique interplay between HBV and hepatic metabolism in greater depth, we systematically reviewed these alterations in metabolic signaling pathways due to HBV infection.

HBV AND GLUCOSE METABOLISM

Glucose homeostasis is regulated by balancing the output and the storage of glucose^[15]. Glucose metabolism in hepatocytes can be divided broadly into two categories: anabolism and catabolism, including gluconeogenesis, glycolysis, aerobic oxidation and the pentose phosphate pathway.

Previous studies indicated that HBV infection could affect either gluconeogenesis or glucose aerobic oxidation. According to the study by Park^[16], hepatitis B virus X protein (HBx) functions as an important positive regulator of gluconeogenesis. In HBx-overexpressing (HBxTg) mice and inducible nitric oxide synthase (iNOS)-knocked out HBxTg mice, increased HBx expression significantly up-regulated the gene expression of hepatic key gluconeogenic enzymes (PEPCK, G6Pase) and the production of hepatic glucose, leading to hyperglycemia and impaired glucose tolerance. These effects are considered to be mediated through the nitric oxide (NO)/JNK pathway. However, other studies have demonstrated that HBV can promote glucose aerobic oxidation. By combining proteomics, metabolomics and molecular biological assays in HepG2.2.15 and HepG2 cell models, Li *et al.*^[17] provided a holistic view of the interplay between host metabolism and HBV. They pointed out that enzymes which regulate the glycolysis pathway, such

as fructose-bisphosphate aldolase, alpha enolase, triosephosphate isomerase, phosphoglycerate kinase 1 and glucose-6-phosphate isomerase, and enzymes involved in the tricarboxylic acid (TCA) cycle, including malate dehydrogenase, citrate synthase and succinate dehydrogenase, are all significantly up-regulated in HepG2.2.15 cells, subsequently leading to elevated levels of corresponding intermediates, such as lactate in glycolysis and fumarate, succinate and 2-oxoglutarate in the TCA cycle. These data suggested that glycolysis and the TCA cycle are stimulated in host cells due to HBV infection. In addition, another study revealed that a HBV pre-S2 mutant could induce aerobic oxidation *via* activation of MTOR signaling, which may contribute to HBV tumorigenesis^[18].

Furthermore, HBV infection could promote the pentose phosphate pathway (PPP). Overexpression of HBx caused the nuclear translocation and activation of NF-E2-related factor 2, resulting in up-regulation of glucose-6-phosphate dehydrogenase, which is the first and rate-limiting enzyme of the PPP converting glucose-6-phosphate into 6-phosphogluconolactone^[19]. Enhancement of the PPP by HBx-mediated elevation of G6PD provided host cells with more ribose for nucleotide biosynthesis to support their proliferation, which might contribute to HBV-associated hepatocarcinogenesis. The change in G6PD was also supported by a systems biology model^[17]. It was reported that G6PD participating in the PPP was markedly increased, accompanied by elevated nucleotide levels, such as AMP, ADP, uridine 59-diphosphate and inosine-59-monophosphate.

HBV AND LIPID METABOLISM

The liver, the main organ for the synthesis and circulation of lipids (*e.g.*, fatty acids, fats, phospholipids and cholesterol), oxidation of fatty acids and the production of ketone bodies, plays an important role in lipid metabolism^[20].

A significant amount of basic research has indicated that HBV infection has an effect on fatty acid metabolism. Many, but not all, studies have shown that HBV can promote the synthesis of fatty acids. Based on HPLC/MS analysis and two-dimensional electrophoresis (2-DE), fatty acid binding 5 and Acyl-CoA binding protein implicated in fatty acid metabolism and synthesis, which can bind Acyl-CoA and fatty acids with high affinity, are markedly increased in hepatitis B virus transgenic mice (HBV-Tg mice)^[21]. HBV-influenced genes in lipid biosynthetic pathways in HBV-Tg mice were identified by cDNA microarray analysis, in which retinol binding protein 1 (RBP1), sterol regulatory element binding protein 2 (SREBP2), ATP citrate lyase and fatty acid synthase (FAS) were all strongly upregulated^[22]. However, in contrast to the above studies, Wang *et al.*^[23] recently proposed that up-regulation of HBx could facilitate fatty acid oxidation (FAO) and subsequently maintain intracellular NADPH

and ATP levels under glucose deprivation, which is of great importance for HCC cell survival under conditions of metabolic stress.

Recently, accumulating evidence from experimental investigations has suggested that HBV infection is a potential trigger of liver steatosis. HBx can induce hepatic steatosis at all aspects, such as increasing fatty acid binding, promoting lipid synthesis and inhibiting secretion of apolipoprotein (Figure 1). Fatty acid binding protein 1 (FABP1), responsible for the uptake, metabolism and transport of long-chain fatty acids (LFA)^[24], plays a key role in intracellular fatty acid utilization and transport^[25]. Forced expression of HBx induced liver steatosis through up-regulation of FABP1, whereas gene silencing of FABP1 blocked lipid accumulation in both *in vivo* and *in vitro* models^[26]. LXR, SREBP1 and PPAR γ are master regulators in hepatic lipogenesis: LXR directly induces expression of SREBP1, which up-regulates lipogenic genes^[27]; activation of LXR also stimulates adipocyte differentiation through induction of PPAR γ expression^[28]. Both are suggested to be of vital importance in hepatic lipid accumulation. Several studies have demonstrated that HBx increased the gene expression and transcriptional activity of LXR-mediated SREBP1 and PPAR γ , thereby inducing the expression of hepatic lipogenic genes (fatty acid synthase, stearoyl-CoA desaturase, acetyl-CoA carboxylase) and adipogenic genes (adipsin, adiponectin, aP2/adipose fatty acid-binding protein), finally accompanied by the accumulation of lipid droplets^[29-32]. Apolipoprotein B (apoB), required for the secretion and assembly of low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL), is assembled into a secretion-competent particle with lipids^[33-35]. It has been reported that HBx mediated aberrantly glycosylated apoB by elevating the expression of N-Acetylglucosaminyltransferase III (GnT-III) resulted in inhibition of apoB secretion as well as intracellular accumulation of cholesterol and triglyceride^[36].

In addition, phospholipid and cholesterol metabolism are also altered by the presence of HBV. Phosphatidylcholine (PC) is a major component of the biological membrane^[37], and acts as a precursor for the synthesis of lipid signaling molecules^[38]. In comparison with HepG2, the key enzymes participating in PC synthesis, such as choline-phosphate cytidyltransferase A, choline kinase α , choline phosphotransferase 1 and choline/ethanolamine phosphotransferase 1 are all up-regulated in HepG2.2.15 cells, consistent with the elevated levels of phosphocholine and reduced levels of choline^[17]. These results strongly indicate that HBV infection can promote the biosynthesis of PC. Cholesterol, a type of lipid different from triglyceride and phospholipid, has two essential metabolic fates: conversion into bile acids or steroid hormones^[39]. HBV-infected humanized mice displayed a significant increase in human genes related to the uptake, biosynthesis, and transcriptional regulation of cholesterol, such as low-density lipoprotein receptor (LDLR), hydroxy-

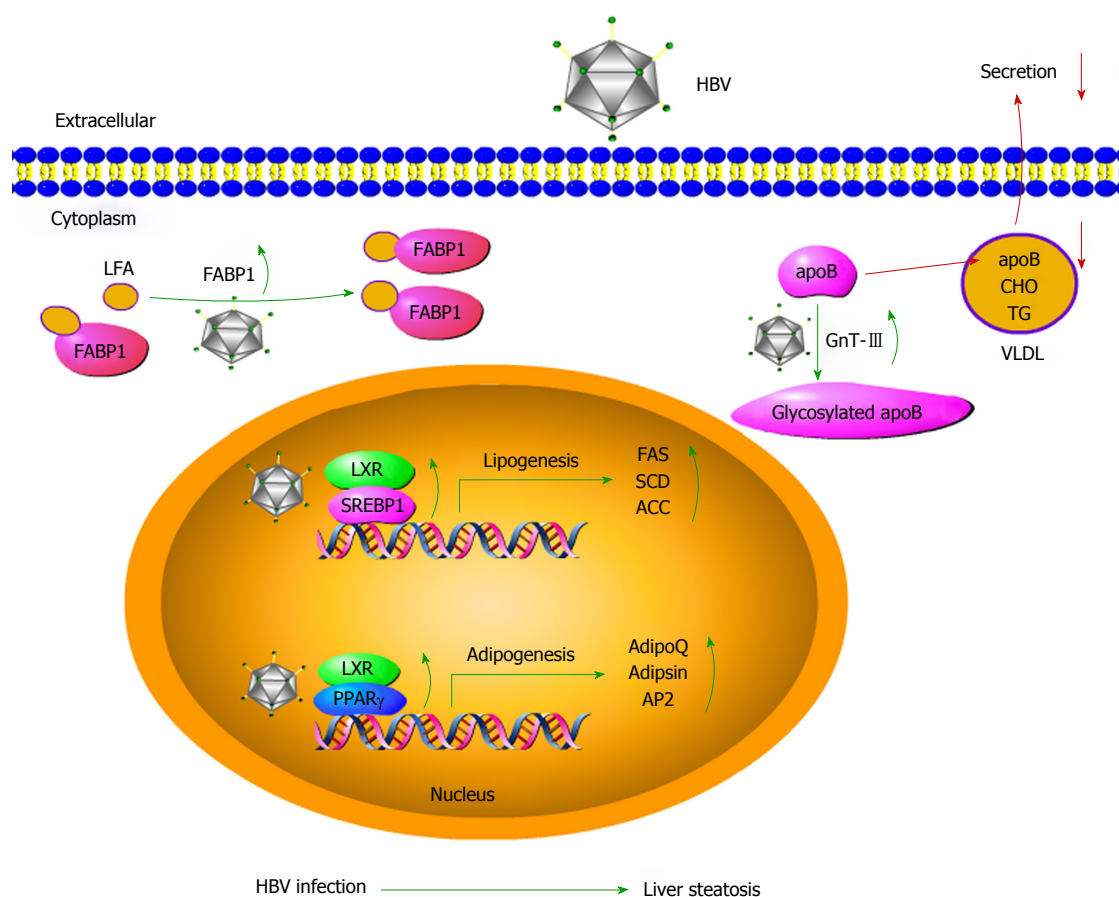


Figure 1 The molecular mechanisms contributing to liver steatosis following hepatitis B virus infection. Hepatitis B virus (HBV) infection can induce the accumulation of lipids *via* three different regulatory mechanisms, including elevated expression of FABP1, up-regulation of LXR, SREBP1 and PPAR γ and increased expression of Gnt-III. On the one hand, up-regulation of FABP1 would increase fatty acid binding and transport. On the other hand, induction of LXR-mediated SREBP1 and PPAR γ would result in increased transcriptional activity of hepatic lipogenic genes (FAS, SCD, ACC) and adipogenic genes (adipoQ, adipsin, AP2). In addition, elevation of Gnt-III would cause glycosylation and dysfunction of apoB, finally leading to reduced secretion of VLDL (containing apoB, CHO and TG). LFA: Long-chain fatty acids; FABP1: Fatty acid binding protein 1; FAS: Fatty acid synthase; SCD: Stearoyl-CoA desaturase; ACC: Acetyl-CoA carboxylase; adipoQ: Adiponectin; AP2: aP2/adipose fatty acid-binding protein; apoB: Apolipoprotein B; Gnt-III: N-Acetylglucosaminyltransferase III; CHO: Cholesterol; TG: Triglyceride; VLDL: Very low density lipoproteins.

methylglutaryl-coenzyme A reductase (HMGCR) and SREBP2^[40]. Another study provided evidence that HBV exacerbated hepatic cholesterol accumulation *via* up-regulation of LDLR and HMGCR in HepG2 cells^[41].

HBV AND NUCLEIC ACID METABOLISM

It is well known that the main function of the nucleotide is the biosynthesis of nucleic acids. Many studies have reported that DNA damage can cause abnormalities in nucleic acid metabolism^[42]. HBV infection also influences this process *via* HBx-induced DNA damage, which may result in the onset of hepatocarcinogenesis^[43]. Thus, identifying the distinguishing nucleic acid metabolites under HBx induction may help to understand the occurrence of HCC. A new study^[44] using a systematic approach combining metabolomics and mRNA microarray analysis indicated that HBx could initially induce DNA damage and then disrupt nucleic acid metabolism, resulting in a significant decrease in DNA damage-related genes (BRCA1, TP53, RPA1, DDB1, TCEA1

and NHEJ1) and intermediates of nucleic acid metabolism (guanosine, inosine and uridine), which in turn blocked DNA repair and probably contributed to the development of HCC.

HBV AND BILE ACID METABOLISM

Bile acid, mainly synthesized in the liver from cholesterol, plays a key role in the digestion and absorption of lipids^[45]. Human NTCP (SLC10A1), located in the basolateral membrane of hepatocytes, functions as a main transporter to mediate entry of bile salts from portal blood into hepatocytes^[46].

Recently, the detection of NTCP, acting as a functional entry receptor for HBV, clearly represents a typical milestone in our knowledge of HBV infection^[47]. HBV exploits NTCP for species-specific entry into hepatocytes^[48]. Hence, emerging evidence demonstrated the probable association between HBV infection and bile acid. Yan *et al.*^[49] showed that the HBV pre-S1 lipopeptide efficiently blocked the uptake of bile salts

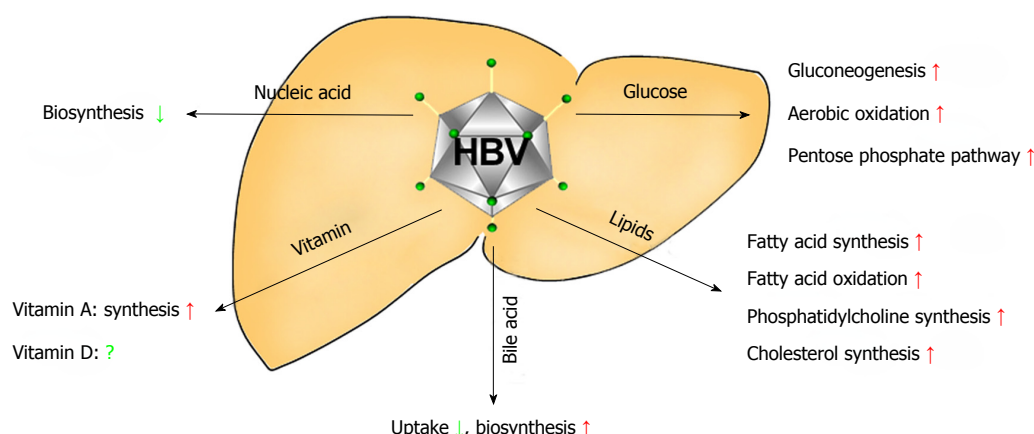


Figure 2 Changes in the hepatic metabolic signaling pathway induced by hepatitis B virus infection. Alterations in related signaling pathways (including glucose, lipids, nucleic acids, bile acids and vitamins) following hepatitis B virus (HBV) infection are marked and highlighted in this figure. The influence of HBV infection on vitamin D metabolism is unclear.

by NTCP, suggesting that HBV infection may limit the physiological function of NTCP. Reduced bile salts could promote compensatory bile acid synthesis to maintain its homeostasis^[50,51]. This compensation was confirmed by the strong induction of hCYP7A1 (the rate-limiting enzyme converting cholesterol to bile acid), decreased FXR (the positive transcription factor of SHP) nuclear translocation and significant reduction of SHP (the corepressor of hCYP7A1 transcription) in human liver-chimeric uPA/SCID mice infected with HBV^[40].

HBV AND VITAMIN METABOLISM

Vitamin A, including retinol, retinal and retinoic acid, plays a critical role in visual function as well as cell growth and differentiation^[52]. Previous data provided evidence that retinoic acid could enhance HBV transcription and replication through activation of RXRa^[53,54]. Most interestingly, another study demonstrated that HBV infection could promote retinol metabolism-related proteins RBP, CRBP1 and ALDH1 as shown by 2-DE and MS/MS analysis^[55]. It is reasonable that more retinol would be pumped into cells and converted into retinoic acid during HBV infection. HBV infection may up-regulate retinoic acid by promoting retinol metabolism and thereby facilitating self-replication through activation of RXRa, leading to an increased risk of liver damage, which was considered a positive feedback^[56].

Vitamin D, including its bioactive vitamin D metabolite [1,25(OH)₂D₃] and stable, easy-to-quantify metabolite (25(OH)D₃), plays an emerging role in metabolic and inflammatory liver diseases^[57]. A study has demonstrated a significant association between low levels of serum 25(OH)D₃ and high HBV DNA levels in CHB patients^[57]. However, the molecular mechanism underlying inverse seasonal fluctuations of HBV DNA and 25(OH)D₃ serum levels still remains to be elucidated.

CONCLUSION

In conclusion, we have systematically outlined the hepatic metabolic responses to HBV infection in this review. According to the above observations, multiple studies combining systematic approaches and molecular biological assays found that, from the molecular mechanism perspective, HBV infection interfered with the hepatic metabolic signaling pathway (Figure 2), including glucose, lipid, nucleic acid, bile acid and vitamin metabolism, ultimately resulting in metabolic derangement. Furthermore, these altered metabolic pathways may also contribute to the pathological processes of other HBV-induced diseases, such as hepatocellular carcinoma. Therefore, in this review, deciphering the molecular mechanisms of the metabolic pathways during HBV infection has shed new light on the pathological processes, and provides a new, revolutionary, potential means of directly fighting against this virus.

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Basic Study

Carbonic anhydrase enzymes II, VII, IX and XII in colorectal carcinomas

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Author contributions: Viikilä P, Parkkila S, Kivelä AJ and Haglund C participated in the design of the study; Clinical and survival data were collected by Koskensalo S and Haglund C; Haglund C collected samples of this study and constructed tissue microarrays; Immunohistochemical staining and light microscopy was performed by Viikilä P; Waheed A, Sly WS, Pastorek J, Pastorekova S and Parkkila S produced and characterized the primary antibodies; Statistical analysis was done by Mustonen H; Viikilä P drafted the first version of the manuscript; all authors were involved in the writing process, read, and approved the final manuscript.

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Abstract

AIM

To investigate expression of four alpha-carbonic anhydrases (CAs) in colorectal carcinomas (CRC) and compare the results with patients' survival.

METHODS

Colorectal carcinoma samples from 539 CRC patients and control tissues were arranged as tissue microarrays and analyzed with antibodies against CA II, CA VII, CA IX, and CA XII. Intensity and extent of staining were both scored from 0 to 3 in each sample. These enzyme expression levels were then correlated to patients' survival and clinicopathological parameters, which were tumor differentiation grade and stage, site of tumor, patients' age, and gender. Kaplan-Meier analysis and Cox regression hazard ratio model were used to analyze survival data.

RESULTS

CA II and CA XII staining intensities correlated with patients' survival in that higher expression indicated poorer prognosis. In Cox regression analysis one unit increase in the CA II intensity increased the hazard ratio to 1.19 fold (CI: 1.04-1.37, $P = 0.009$). A significant correlation was also found when comparing CA XII staining intensity with survival of CRC patients (HR = 1.18, 95%CI: 1.01-1.38, $P = 0.036$). The extent of CA XII immunostaining did not correlate to the patients' survival ($P = 0.242$, Kaplan-Meier analysis). A significant interaction between age group and extent of the CA II staining was found. Increased extent of CA II had a significant hazard ratio among patients 65 years and older (1.42, 95%CI: 1.16-1.73, $P = 0.0006$). No correlations were found between CA VII (intensity $P = 0.566$, extent $P = 0.495$, Kaplan-Meier analysis), or CA IX (intensity $P = 0.879$, extent $P = 0.315$, Kaplan-Meier analysis) immunostaining results and survival, or the other parameters.

CONCLUSION

The present findings indicate that CA II and CA XII could be useful in predicting survival in CRC.

Key words: Biomarker; Carbonic anhydrase; Colorectal cancer; Immunohistochemistry; Prognosis; Survival

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Core tip: Our aim was to investigate expression of four alpha-carbonic anhydrases (CAs) in colorectal carcinomas (CRC) and compare the results with patients' survival. CRC samples were arranged as tissue microarrays and analyzed with antibodies against CA II, CA VII, CA IX, and CA XII. Enzyme expression levels were correlated to patients' survival and clinicopathological parameters. CA II and CA XII staining intensities correlated with patients' survival in that higher expression indicated poorer prognosis. The present findings indicate that CA II and CA XII could be useful in predicting survival in CRC.

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INTRODUCTION

Colorectal cancer (CRC) is the most common malignancy of the gastrointestinal (GI) tract; it is the second most common cancer in women and third in men^[1]. Its incidence shows substantial geographical variation, resulting in a 10-fold difference between some countries^[1]. The high number of patients with CRC, precancerous lesions or polyps causes significant challenges to national healthcare systems, their chances to recognize tumors at an early stage, as well as possibilities to offer the most effective treatment. According to recent data the incidence of CRC is rising in many traditionally low-incidence countries like Japan, Korea, China, and Eastern Europe, which is thought to be a result of cultural and dietary changes towards a Western lifestyle^[1]. Even though the overall incidence is increasing, at the same time some positive development has occurred in many previous high-risk countries due to more effective diagnostics and treatment.

CRC usually develops from precursor lesions, *i.e.*, colonic adenomas^[2]. The prevalence of adenomas clearly increases by age. Postmortem studies indicate that 30%-40% of individuals from Western countries have adenomas, most of them asymptomatic^[3]. A majority of these are benign and never turn malignant^[2]. In familial syndromes, younger patients develop adenomas which proceed to cancer with a much higher frequency^[2-4]. Removal of precancerous lesions at colonoscopy prevents them from proceeding to cancer. Thus, early diagnosis and complete removal of precancerous lesions are key factors for successful outcome.

The diagnostic arsenal in CRC includes several clinicopathological parameters, which can be utilized to determine the malignancy and prognosis. They include tumor stage according to the TNM (tumor, nodes, metastasis), Dukes or Astler-Coller classification, as well as tumor grade, microsatellite instability, and molecular markers, recently reviewed by Marzouk and Schofield^[5]. The intensively studied molecular markers include such as tumor suppressor gene p53 and its mutations as well as antigen Ki-67^[6,7]. However, new biomarkers are urgently needed to predict survival and improve stratification of CRC patients for different treatment options.

Carbonic anhydrases (CAs) constitute a group of zinc-binding enzymes, which catalyze the reversible hydration of CO₂ to bicarbonate. This reaction is crucial for maintenance of pH homeostasis of the body. Through this chemical reaction they are involved in several downstream physiological processes, such as

Table 1 Patient characteristics

	<i>n</i> (%)
Total	539 (100)
Sex	
Male	294 (54.5)
Female	245 (45.5)
Age	
< 65 yr	231 (42.9)
≥ 65 yr	308 (57.1)
Tumor location	
Right side of colon	157 (29.1)
Left side of colon	138 (25.6)
Rectum	244 (45.3)
Stage	
Dukes A	82 (15.2)
Dukes B	191 (35.4)
Dukes C	136 (25.2)
Dukes D	130 (24.1)
Differentiation (grade)	
High	19 (3.5)
Medium high	352 (65.3)
Medium low	141 (26.2)
Low	27 (5.0)

bone resorption, vision, and production of saliva, bile, pancreatic juice and gastric juice^[8-10]. The mammalian alpha-CA family includes 16 known isoforms of which 15 can be found in humans. These isoforms show marked differences in their kinetics and cellular and subcellular distributions^[8,9].

Under hypoxic conditions cells produce acidic metabolic products *via* anaerobic glycolysis. This pathway is inhibited in the presence of enough oxygen. Notably, tumor cells have a tendency to upregulate glucose intake and increase the rate of anaerobic glycolysis even when the amount of oxygen is sufficient^[11]. Tumor cells need CA enzymes and many other proteins, such as ion transporters, to maintain neutral intracellular pH^[12]. During this process extracellular pH decreases, which in turn, disturbs physiological processes of the surrounding normal tissue and promotes cancer growth^[11,13]. Indeed, increased glucose intake and hypoxia are often linked to more aggressive and invasive tumor growth, signs that correlate with poor prognosis^[11]. It has been suggested that partial hypoxia may contribute to cell selection, favoring a shift from a pre-malignant phenotype to more malignant forms, in which the oxygen free metabolism plays a major role in making it possible for cells to survive in challenging hypoxic environments^[11].

During the last 20 years, CA proteins have been studied as potential markers for various cancers. Cytosolic CA II is the most widely expressed isoform in normal tissues, such as gastric, pancreatic, biliary, and intestinal epithelia^[9,14]. It is often absent or only weakly expressed in malignant tumors. Recently, CA II was shown to be highly overexpressed in gastrointestinal stromal tumors, and it was suggested as a potential biomarker for this mesenchymal tumor type^[15]. CA VII, another cytosolic isozyme, shows a more restricted tissue distribution than CA II. It is predominantly expressed

in the brain, where it contributes to bicarbonate-driven GABAergic excitation^[16]. A recent study showed that CA VII is overexpressed in glioblastomas, suggesting that it may represent another tumor-associated CA isoform^[17].

CA IX has attracted lots of attention, because its expression is limited to few normal tissues, such as gastric, intestinal and gall bladder epithelia, but it is highly overexpressed in hypoxic tumors^[9,18,19]. CA XII is another isoform, which is overexpressed in several cancers, even though it is also present in various normal tissues. It has been demonstrated to be present in both normal intestinal epithelium and malignant colorectal tumors^[9,19,20]. CA IX and XII are known to be regulated *via* von Hippel Lindau / hypoxia inducible factor pathway^[21].

The aim of this study was to investigate the expression of isozymes CA II, CA VII, CA IX, and CA XII in CRC. The immunohistochemical expression levels were correlated to clinicopathological data. Our results show that both CA II and CA XII staining intensities correlate with survival rate of CRC patients, suggesting a potential role of these enzymes as prognostic biomarkers.

MATERIALS AND METHODS

Patients

In total, 840 patients underwent surgery for CRC at Helsinki University Hospital during years 1983-2001. Tissue specimens and clinical data from 645 patients were available for our study. These tumors were classified with Dukes classification which was a standard classification system during the sample collection period. No information of TNM - classification was available for the analysis. The Ethical Committee of Helsinki University Hospital (Dnro 226/E6/2006) and National Supervisory Authority for Welfare and Health (Dnro 10041/06.01.03.01/2012) granted permission for the use of these samples. Survival data were available for all patients and obtained from patient records, the Finnish Cancer Registry and Statistics Finland. The clinicopathological characteristics of the patients are described in Table 1.

Immunohistochemical analysis

Tumor samples were arranged as tissue microarrays. Three parallel series included one tumor sample each taken with a 1 mm needle. Of 645 patient samples we obtained, 106 ended up with no scoring results, because samples were washed out or displaced during the staining process. The remaining 539 samples were considered representative enough to be analyzed and scored. Each microscope slide contained four spots of normal human pancreas as positive controls. Five µm sections of formalin-fixed paraffin embedded tumor specimens were deparaffinised and immunostained with rabbit anti-human CA II, CA VII, and CA XII sera or with monoclonal anti-human CA IX antibody (M75). These antibodies have been previously

Table 2 Summary of carbonic anhydrases stainings

	Mean	SD	Median	Mode	25%	75%
CA II epithelium intensity	1.8	1.1	2	3	1	3
CA II epithelium extent	2.2	1.1	3	3	2	3
CA II endothelium	0.9	0.3	1	1	1	1
CA VII epithelium intensity	1.4	1	1	1	1	2
CA VII epithelium extent	2	1.2	3	3	1	3
CA VII stromal intensity	2.8	0.5	3	3	3	3
CA VII stromal extent	2.8	0.5	3	3	3	3
CA IX epithelium intensity	1.7	1.2	2	3	0	3
CA IX epithelium extent	1.6	1.2	2	3	0	3
CA XII epithelium intensity	1.5	0.9	1	2	1	2
CA XII epithelium extent	2	1.1	2	3	1	3

utilized in numerous studies and are specific for each isozyme^[22-25]. Immunoperoxidase staining was performed in an automated Lab Vision Autostainer 480 (LabVision Corporation, Fremont, CA, United States) by Power Vision+ Poly-HRP Immunohistochemistry kit reagents (ImmunoVision Technologies Co) including the following steps: (1) rinsing in wash buffer; (2) treatment in 3% H₂O₂ in ddH₂O for five minutes and rinsing with wash buffer; (3) blocking with cow colostrum diluted 1:2 in Tris-buffered saline containing 0.05% Tween-20 for 30 min and rinsing in wash buffer; (4) incubation with primary antibody (polyclonal antibodies diluted 1:2000 and monoclonal M75 diluted 1:100) for 30 min; (5) rinsing in wash buffer three times for five minutes; (6) incubation in poly-HRP-conjugated anti-rabbit/mouse IgG for 30 min and rinsing in wash buffer three times for five minutes; (7) incubation in DAB (3,3'-diaminobenzidine tetrahydrochloride) solution (one drop of DAB solution A and one drop of DAB solution B in 1 mL of ddH₂O) for six minutes and rinsing in ddH₂O; (8) CuSO₄ treatment for five minutes to enhance the signal and rinsing in ddH₂O; (9) treatment with hematoxylin for one minute; and (10) rinsing with ddH₂O. All procedures were performed at room temperature. The mounting of the sections was performed using Entellan Neu (Merck; Darmstadt, Germany) and was finally examined and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss; Göttingen, Germany). The intensity of the staining was scored on a scale of 0 to 3 as follows: 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction. The extent of the staining was scored as 1 when 1%-10% of the cells stained, 2 when 11%-50% of the cells stained, and 3 when 51%-100% of the cells stained. A negative score (0) was given to tissue sections that had no evidence of specific immunostaining. In addition to the analysis of tumor cells, CA II immunostaining was also evaluated in endothelial cells and CA VII in tumor stroma, because these enzymes showed significant staining reactions in these specific locations.

Statistical analysis

All statistical analyses were performed by an experienced biostatistician (H. Mustonen). Results are

announced as number of patients and percentage of patients, the Kaplan-Meier mean survival time with 95%CI, and the Cox regression hazard ratios with their 95%CI. *P* values < 0.05 were considered statistically significant. Two-tailed tests were used.

Kaplan-Meier analysis and Cox regression hazard ratio model were used to analyze survival data. The assumption of constant hazard ratios over time was tested by including a time dependent covariate for each testable variable. Because neither Dukes classification nor histological differentiation grade status followed the Cox model assumption, stratified analyses for Dukes stage and differentiation grade were used. For statistical analyses, Dukes stage A and B, as well as C and D were combined to reduce the number of groups to two. The same was done for tumor differentiation status; low and medium low differentiations were combined as well as medium high and high differentiations. In the Cox proportional hazards model we included variables for age, sex, and site of the tumor. Separate analyses were made for each CA staining. If a significant interaction with CA staining and another parameter were found, a split analysis was performed.

In analysis, male patients were compared to females and 65 years-old or older patients were compared to younger ones. Tumor location was also considered in the analysis: right side of the colon including the caecum, ascending colon and right flexure versus left side of the colon, including the left flexure, descending colon, sigmoid colon and rectum. Mid transverse tumors were excluded from the comparison.

When analyzing the expression of each CA enzyme, level 0 was considered the baseline, and the announced hazard ratio rises exponentially every time the scoring value rises one unit. Statistical analyses were performed by SPSS v 21© (IBM corp, New York).

RESULTS

Four CA isozymes, CA II, VII, IX, and XII were selected for the immunohistochemical analysis of colorectal cancer and the summary of these results is presented in Table 2. Figure 1 presents representative images

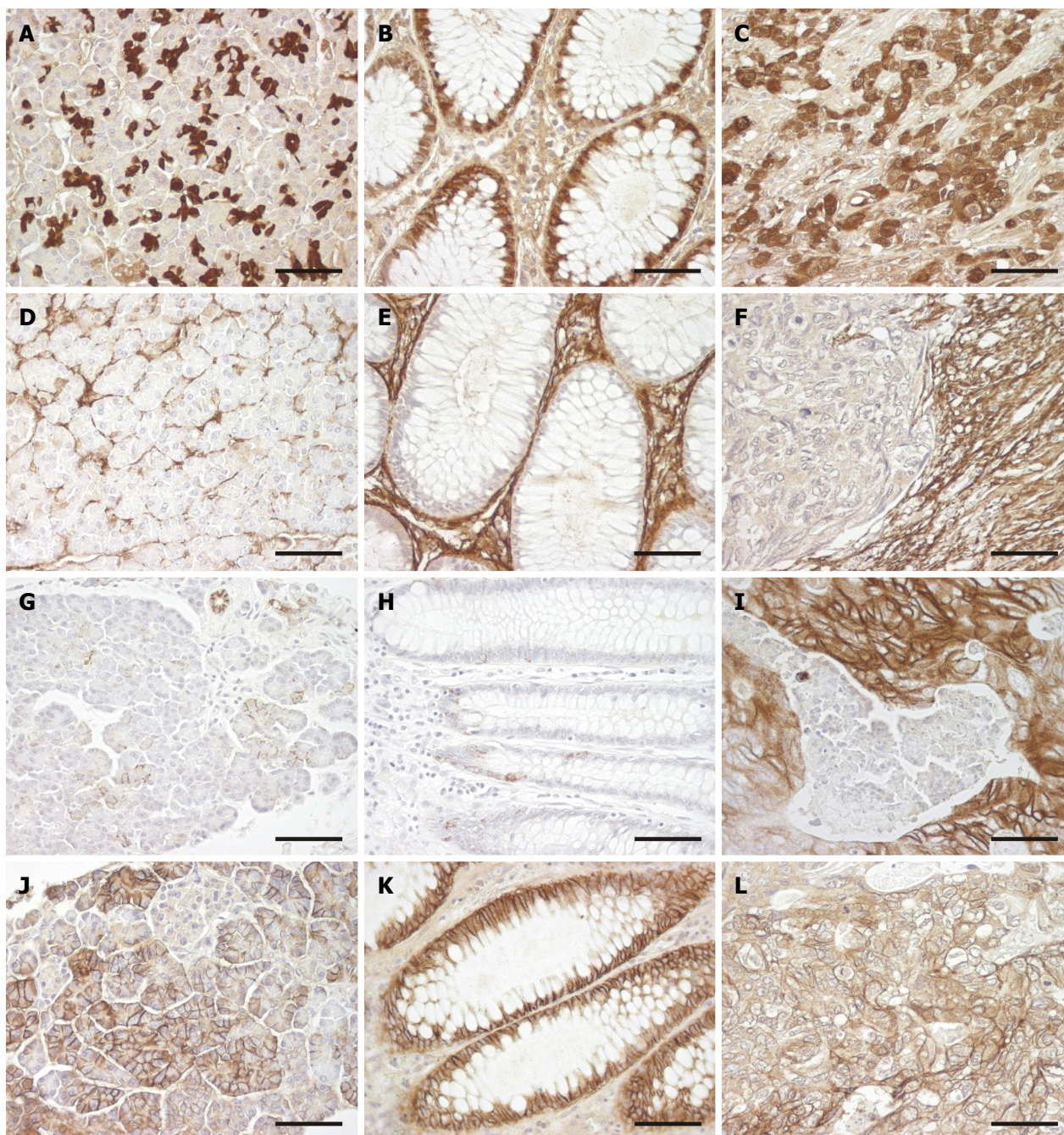


Figure 1 Immunohistochemical stainings of carbonic anhydrases II, VII, IX and XII. CA II (A, B, C), CA VII (D, E, F), CA IX (G, H, I), and CA XII (J, K, L) are shown in specimens of normal pancreas (A, D, G, J), colon (B, E, H, K) and colorectal carcinoma (C, F, I, L). The normal pancreas and colon sections were used for control purposes. CA II was located to the pancreatic ducts and centroacinar cells. In normal colon the staining is strongest in the enterocytes of deep crypts. In colorectal cancer, CA II is strongly expressed in cancer cells. CA VII stains stromal tissue in all specimens, while the cancer cells show only minimal or no staining. CA IX antibody weakly stains ductal and some acinar cells of pancreatic tissue and also basolateral cell membranes in basal parts of colonic crypts. In colorectal cancer, CA IX is strongly expressed in malignant cells, whereas the necrotic part is negative. CA XII antibody stains basolateral membranes of pancreatic acinar cells and colonic enterocytes, and malignant cells of colon carcinoma. Original magnifications $\times 400$, Bars $50\ \mu\text{m}$.

of positive immunostaining of each isozyme in CRC specimens. Normal colon and pancreas specimens are shown as positive controls. Figure 2 shows Kaplan-Meier plots for the staining intensity and extent of each isozyme. In Kaplan-Meier analysis, there was a significant decrease in survival as the intensity of CA II increased. The mean survival time decreased from 17.7 years (95% CI, 14.8-20.6) to 12.3 years (95% CI:

10.5-14.2) when CA II intensity increased from 0 to 3. The extent of CA II staining showed a similar trend ($P = 0.022$). Correspondingly, the mean survival time decreased from 18.3 years (95% CI: 15.5-21.1) to 13.5 years (95% CI: 12.0-15.0).

In Cox regression analysis, the most significant results were found for CA II staining intensity as shown in Table 3. One unit increase in the CA II

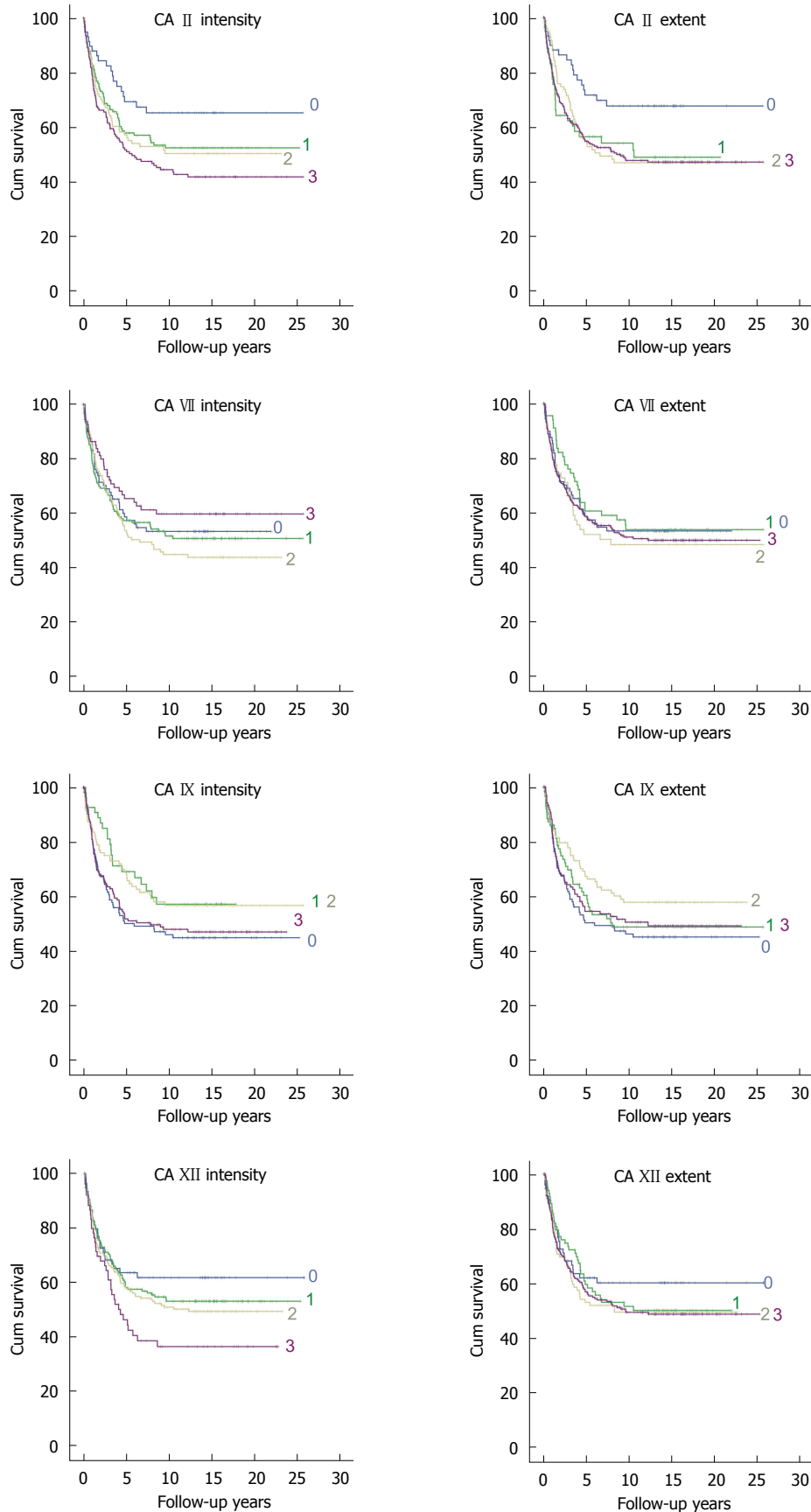


Figure 2 Effect of CA II, CA VII, CA IX and CA XII expression on survival of colorectal patients. Survival of 539 colorectal cancer patients operated between 1983-2001 in Helsinki University Hospital was recorded and the expression of carbonic anhydrases (CA II, CA VII, CA IX and CA XII) was evaluated from immunohistochemical stainings of tumor specimens. The effects of CA expressions on survival were analyzed by Kaplan-Meier analyses. Both CA II staining intensity ($P = 0.004$) and extent ($P = 0.022$) correlated with the patient survival. A weak correlation was also found between the CA XII staining intensity and survival ($P = 0.020$).

Table 3 Multivariate Cox regression analysis results of selected parameters

	Hazard ratio	95%CI lower	95%CI upper	P value
CA II epithelium intensity analysis				
CA II epithelium intensity	1.19	1.04	1.37	0.0092
Sex (men <i>vs</i> females)	1.20	0.92	1.58	0.1777
Age over 65 yr	1.78	1.35	2.35	$P < 0.0001$
Side of colon (sin <i>vs</i> dex)	1.17	0.86	1.58	0.3170
CA II epithelium extent analysis, age \leq 65 yr				
CA II epithelium extent	0.88	0.73	1.06	0.1742
Sex (men <i>vs</i> females)	0.99	0.65	1.51	0.9564
Side of colon (sin <i>vs</i> dex)	1.63	0.97	2.74	0.1869
CA II epithelium extent analysis, age > 65 yr				
CA II epithelium extent	1.42	1.16	1.73	0.0006
Sex (men <i>vs</i> females)	1.44	1.01	2.05	0.0435
Side of colon (sin <i>vs</i> dex)	0.92	0.63	1.35	0.6764
CA XII epithelium intensity analysis				
CA XII epithelium intensity	1.18	1.01	1.38	0.0360
Sex (men <i>vs</i> females)	1.23	0.94	1.60	0.1301
Age over 65 yr	1.67	1.27	2.19	0.0002
Side of colon (sin <i>vs</i> dex)	1.09	0.81	1.47	0.5571

Regression analyses are stratified by Dukes and differentiation status. Separate analyses were done for each carbonic anhydrases (CAs) staining. Due to interaction between age and epithelium extent, the analysis was split in different age groups.

Table 4 Carbonic anhydrases VII and Dukes stage

CA VII epithelial extent	Dukes A count	%	B count	%	C count	%	D count	%
0	15	16.1%	36	38.7%	25	26.9%	17	18.3%
1	14	19.7%	31	43.7%	14	19.7%	12	16.9%
2	10	15.2%	23	34.8%	14	21.2%	19	28.8%
3	28	11.4%	85	34.6%	67	27.2%	66	26.8%

$P = 0.013$, linear by linear association test.

intensity increased the age, sex, Dukes, differentiation, and tumor side corrected hazard ratio to 1.19 fold (95%CI: 1.04-1.37, $P = 0.009$). However, no significant interaction was found between CA II intensity and age, sex, or location of tumor. When extent of the CA II staining was analyzed a significant interaction between age group and extent of the CA II staining was found. Therefore, the analysis was split between the age groups. This revealed that increased extent of CA II had a significant hazard ratio among patients 65 years and older (1.42, 95%CI: 1.16-1.73, $P = 0.0006$). Notably, in addition to the actual tumor cells, CA II -positive staining was often induced in the endothelium of tumor capillaries. In Kaplan-Meier analysis, vascular endothelial staining of CA II did not show any significant correlation to survival ($P = 0.676$).

Epithelial CA VII immunostaining showed no correlation to patients' survival (Figure 2). In Kaplan-Meier survival analysis, P values were 0.566 for staining intensity and 0.495 for extent. Negative results were also observed for stromal staining ($P = 0.816$ for intensity; $P = 0.591$ for extent). There was a significant correlation ($P = 0.013$) between epithelial staining extent and Dukes classification. These results are shown in Table 4. No significant correlation was found between epithelial or stromal CA

VII immunostaining and other parameters.

In Kaplan-Meier analysis no significant correlation was found between survival and intensity ($P = 0.879$) or extent ($P = 0.315$) of CA IX immunostaining. Additionally, no correlation was found when the CA IX immunostaining was compared with the other tumor or clinical parameters.

In Kaplan-Meier analysis, CA XII was another isozyme showing a significant correlation to survival. The mean survival time decreased from 16.5 years (95%CI: 13.7-19.2) to 9.9 years (95%CI: 7.3-12.4) as the intensity of CA XII increased from 0 to 3. In Cox regression analysis, a significant correlation was found when comparing CA XII staining intensity with survival of CRC patients (HR = 1.18, 95%CI: 1.01-1.38, $P = 0.036$; Table 3). There was no significant interaction between CA XII intensity and age, gender or location of the tumor. The extent of CA XII immunostaining did not correlate to the patients' survival ($P = 0.242$, Kaplan-Meier analysis). CA XII staining extent did not either correlate with clinicopathological parameters.

In general, patients 65 years and older had a significantly increased risk for poorer survival (HR = 1.78, 95%CI: 1.35-2.34, $P < 0.0001$ in CA II epithelium intensity analysis and 1.67, CI: 1.27-2.19, $P = 0.0002$ in CA XII epithelium intensity analysis).

DISCUSSION

In our study, the expression of carbonic anhydrase isozymes CA II and CA XII correlated with patients' survival. Our results suggest that these proteins might have a value in the assessment of CRC patients, specifically when selecting patients for a more aggressive cancer treatment. Patients with a better prognosis can be treated less aggressively, which diminishes side effects and increases quality of life. The obtained hazard ratios were relatively modest, and thus the results need to be interpreted with caution. According to our results, CA IX has no diagnostic or prognostic role in CRC, even though it has been considered a promising biomarker for several other cancers^[26]. This finding highlights the unique biology of CRCs in terms of tumor development process.

The role of different CA isozymes in cancer has been under intensive research during the last decade. Studies have been focused on the association between expression of CAs and tumor aggressiveness and patient survival, on their role in tumor metastasis, as well as on their potential role as targets of anticancer drugs^[13,27-29]. CA isozymes have shown abnormal expression in various malignant tumors compared to corresponding normal cells and tissues^[11,13,17,29-31]. In one study, CA IX was shown to be diffusely expressed in CRC, whereas normal or adenomatous mucosa showed a more limited distribution^[18]. Ki-67 immunostainings confirmed that CA IX was expressed in areas with high proliferative activity. Similarly, CA XII showed a more diffuse immunostaining reaction in CRC compared to normal colon or adenomas^[20]. Cytosolic CA II isozyme has a different distribution. It is highly expressed in several normal gastrointestinal tract tissues, such as gastric and intestinal mucosa^[14], but is clearly downregulated in most colorectal tumors^[32]. The same phenomenon may also occur in the case of the other cytosolic CAs^[33]. According to Birkenkamp-Demtroder's study^[34], CA VII expression was found to be 4-fold downregulated in sigmoid or rectosigmoid carcinomas compared to the normal tissue. Recently, Niemelä *et al.*^[35] reported cDNA microarray expression of all CAs, except for CA XIII, in normal and malignant colorectal specimens. The fold-changes for our target CA isozymes were: CA II [-7.4 (normal mucosa vs sporadic carcinoma)], CA VII (-4.3), CA IX (+2.4) and CA XII (-3.2). In our Figure 1 increased expression is clearly demonstrated for CA IX, whereas the interpretation is more difficult for the other isozymes because of high staining intensities in both normal and tumor tissues.

Even though the pathogenesis of colorectal cancer has been intensively studied during the last decades, there are still molecular mechanisms that clearly warrant more research. For example, it was recently shown that polyps with any advanced neoplastic features are smaller in the right side of the colon

than in the left side, and the gene expression is also different^[34,36]. In our study, however, the reactivity of CA enzymes showed no correlation to the tumor location.

The main clinicopathological factors affecting colorectal cancer patients' survival are tumor grade, resection margins, and presence or absence of lymph node metastases^[3]. Other factors with prognostic significance include tumor budding, micrometastases, peritoneal carcinomatosis, lymphatic, perineural and venous invasion, and histological properties including level of invasion^[5]. In Marzouk and Schofield's review^[5], a number of potential prognostic molecular markers, such as microsatellite instability, BRAF mutation, KRAS mutation, PIK3CA mutations, and PTEN deletion, were evaluated. 18q deletions and thymidylate synthase expression have been associated with unfavorable prognosis and tumor recurrence^[5]. Even though many of these molecular markers have been used when choosing patients with metastatic disease for chemotherapy^[5], new markers are still needed to distinguish different cancer types or to stratify patients for personalized chemotherapy according to the cancer properties. Further studies are needed to define the clinical value of CA II and CA XII staining for preoperative evaluation and for comparison of these markers in colorectal cancer.

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COMMENTS

Background

Colorectal cancer (CRC) is the most common malignancy of the gastrointestinal tract. The high number of patients with CRC, precancerous lesions or polyps causes significant challenges to healthcare systems, their chances to recognize tumors at an early stage, as well as possibilities to offer the most effective treatment. Tumor cells need carbonic anhydrases (CAs) and many other proteins, to maintain neutral intracellular pH. During tumor growth extracellular pH decreases and disturbs physiological processes of the surrounding normal tissue and promotes cancer growth. This is why CA proteins have been studied as potential markers for various cancers.

Research frontiers

CA II and CA XII staining intensities correlated with colorectal cancer patients' survival in that higher expression indicated poorer prognosis.

Innovations and breakthroughs

The correlation of both CA II and CA XII staining results with survival of colorectal cancer patients was a novel observation. Surprisingly, CA IX immunostaining results did not show any correlation to patients' prognosis, even though this enzyme is widely considered a potential predictive marker in several other tumor categories.

Applications

These results suggest that CA II and CA XII proteins might be valuable in

assessment of CRC patients, specifically when selecting patients for a more aggressive cancer treatment. Patients with a better prognosis can be treated less aggressively, which diminishes side effects and increases quality of life.

Terminology

CAs are a group of zinc-binding enzymes, which catalyze the reversible hydration of CO₂ to bicarbonate.

Peer-review

Biomarkers for colorectal Cancer are still under intense investigation and novel predictive and prognostic markers are urgently needed. The authors investigate the Protein Expression Levels of several CA in a large series of colorectal cancer and conclude that CA II and CA XII could be useful prognostic markers.

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Basic Study

Hepatocyte isolation from resected benign tissues: Results of a 5-year experience

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Abstract

AIM

To analyze retrospectively a 5-year experience of human hepatocyte isolation from resected liver tissues with benign disease.

METHODS

We established a method of modified four-step retrograde perfusion to isolate primary human hepatocytes. Samples were collected from the resected livers of patients with intrahepatic duct calculi ($n = 7$) and liver hemangioma ($n = 17$). Only the samples weighing ≥ 15 g were considered suitable for hepatocyte isolation. By using the standard trypan blue exclusion technique, hepatocyte viability and yield were immediately determined after isolation.

RESULTS

Twenty-four liver specimens, weighing 15-42 g, were immediately taken from the margin of the removed samples and transferred to the laboratory for hepatocyte isolation. Warm ischemia time was 5-35 min and cold ischemia time was 15-45 min. For the 7 samples of intrahepatic duct calculi, the method resulted in a hepatocyte yield of $3.49 \pm 2.31 \times 10^6$ hepatocytes/g

liver, with $76.4\% \pm 10.7\%$ viability. The 17 samples of liver hemangioma had significantly higher yield of cells ($5.4 \pm 1.71 \times 10^6$ cells/g *vs* $3.49 \pm 2.31 \times 10^6$ cells/g, $P < 0.05$) than the samples of intrahepatic duct calculi. However, there seems to be no clear difference in cell viability ($80.3\% \pm 9.67\%$ *vs* $76.4\% \pm 10.7\%$, $P > 0.05$). We obtained a cell yield of $5.31 \pm 1.87 \times 10^6$ hepatocytes/g liver when the samples weighed > 20 g. However, for the tissues weighing ≤ 20 g, a reduction in yield was found ($3.08 \pm 1.86 \times 10^6$ cells/g *vs* $5.31 \pm 1.87 \times 10^6$ cells/g, $P < 0.05$).

CONCLUSION

Benign diseased livers are valuable sources for large-number hepatocyte isolation. Our study represents the largest number of primary human hepatocytes isolated from resected specimens from patients with benign liver disease. We evaluated the effect of donor liver characteristics on cell isolation, and we found that samples of liver hemangioma can provide better results than intrahepatic duct calculi, in terms of cell yield. Furthermore, the size of the tissues can affect the outcome of hepatocyte isolation.

Key words: Human hepatocyte; Primary hepatocyte; Cell isolation; Benign liver disease; Hepatocyte isolation

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Core tip: We retrospectively analyzed a 5-year experience of human hepatocyte isolation from surgically-resected normal tissues and established an efficient technique for a special kind of liver samples for large-scale human hepatocyte isolation. Our study represents the largest number of primary human hepatocytes isolated from resected specimens from patients with benign liver disease. We evaluated the effect of donor liver characteristics on cell isolation, and we found that samples of liver hemangioma can provide better results than intrahepatic duct calculi, in terms of cell yield. Furthermore, the size of the tissues can affect the outcome of hepatocyte isolation.

Meng FY, Liu L, Liu J, Li CY, Wang JP, Yang FH, Chen ZS, Zhou P. Hepatocyte isolation from resected benign tissues: Results of a 5-year experience. *World J Gastroenterol* 2016; 22(36): 8178-8186 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i36/8178.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i36.8178>

INTRODUCTION

Demands for primary human hepatocytes for basic research and therapeutic applications are continuously increasing^[1-3]. Large quantities of primary human hepatocytes can be isolated mainly from two sources: discarded liver transplants and liver specimens

obtained during partial hepatectomy^[4,5]. Compared to organs from discarded liver transplants, the latter are easily accessible and more frequently available. Furthermore, resected liver donations are usually planned and often occur during normal working hours. Nevertheless, surgically-resected tissue is of varying quality, which can affect the yield and viability of isolated hepatocytes^[6]. It is important to standardize the use of the surgical specimens to maximize the availability of high-quality hepatocytes.

Some authors have previously suggested protocols for human hepatocyte isolation from surgically-resected liver tissues^[5,7,8]. However, the majority of these groups have used tissues from malignant tumors^[9]. Often, totally healthy tissues are needed for clinical application of hepatocyte transplantation, artificial liver, and hepatocyte immortalization^[10,11]. There are still no systematic studies in the literature that have investigated resected specimens from patients with benign liver disease for large-scale hepatocyte isolation. Therefore, available data about the effect of the donor liver on isolated hepatocyte yield are scarce.

To address these issues, we retrospectively analyzed a 5-year experience of human hepatocyte isolation from surgically-resected normal tissues and evaluated the effect of donor liver characteristics on hepatocyte isolation outcome. Our study, presented herein, represents the largest number of primary human hepatocytes isolated from resected specimens from patients with benign liver disease. We established an efficient technique for a special kind of liver samples for large-scale human hepatocyte isolation.

The aim of our study was to rescue resected healthy liver tissues that would have been otherwise discarded, for hepatocyte isolation. We also compared two different kinds of liver samples and their results in different cell yield and viability.

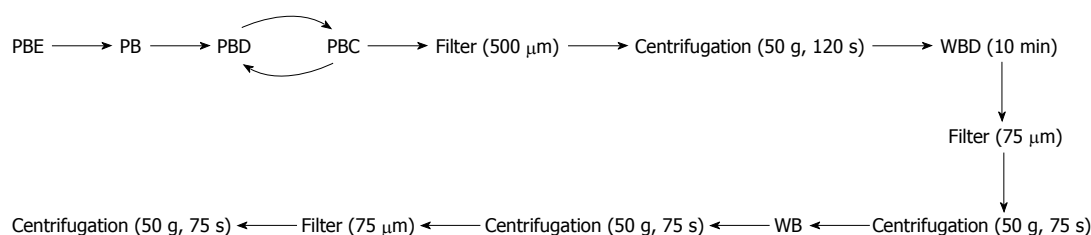
MATERIALS AND METHODS

Surgery and tissue collection

We chose patients with benign liver disease, intrahepatic duct calculi and liver hemangioma, treated in our hospital. Following our hospital's institutional and ethical guidelines, and after obtaining tissue donors' consent, samples weighing ≥ 15 g were collected from 24 patients undergoing partial hepatectomy, including 7 liver intrahepatic duct calculi and 17 liver hemangioma (Table 1). To avoid unnecessary damage to the hepatocytes, continuous clamping was applied in all 24 cases, with a short intraoperative warm ischemia time (WIT) (5-35 min). After the liver tissues (15-42 g) were resected from the abdominal cavity, they were immediately placed into ice-cold Ringer's lactate solution, under sterile conditions. The tissues were transferred directly to the laboratory for hepatocyte isolation, with a limited cold ischemic time (15-45 min).

Table 1 Patient profile and isolated human hepatocyte viability, total cell yield and hepatocyte yield

No.	Sex	Age (yr)	Blood group	Disease	Tissue weight (g)	Warm ischemia time (min)	Hepatocyte ($\times 10^5/\text{g}$)	Viability (%)
1	Male	48	O ⁺	Hemangiomas	15	30	28.00	62
2	Male	52	A ⁺	Hemangiomas	18	25	20.56	73
3	Female	23	A ⁺	Calculus	21	25	12.86	69
4	Male	55	O ⁺	Hemangiomas	26	30	18.08	65
5	Female	45	A ⁺	Hemangiomas	27	25	51.85	76
6	Female	36	B ⁺	Hemangiomas	32	15	65.63	85
7	Male	47	AB ⁺	Calculus	38	10	71.05	92
8	Male	42	A ⁺	Hemangiomas	42	10	54.76	86
9	Male	52	A ⁺	Hemangiomas	20	5	63.30	87
10	Male	63	O ⁺	Hemangiomas	33	25	72.11	93
11	Female	46	O ⁺	Hemangiomas	27	20	43.70	68
12	Male	57	A ⁺	Hemangiomas	30	35	55.21	75
13	Male	44	B ⁺	Calculus	25	15	63.81	84
14	Male	61	A ⁺	Calculus	17	5	25.20	86
15	Female	57	A ⁺	Hemangiomas	32	7	62.50	89
16	Male	27	A ⁺	Hemangiomas	27	15	52.65	76
17	Female	55	B ⁺	Calculus	23	7	32.07	66
18	Male	61	O ⁺	Hemangiomas	31	25	68.80	90
19	Male	37	B ⁺	Hemangiomas	37	21	72.22	92
20	Male	65	B ⁺	Calculus	20	15	17.07	72
21	Male	79	O ⁺	Calculus	26	20	22.51	66
22	Male	47	O ⁺	Hemangiomas	35	5	67.72	89
23	Male	51	B ⁺	Hemangiomas	24	12	59.27	77
24	Male	67	O ⁺	Hemangiomas	30	17	62.25	82

**Figure 1 Flow diagram of the preparation of isolated human hepatocytes with a modified four-step retrograde perfusion technique.** Buffers: PBE: Perfusion buffer with EDTA; PB: Perfusion buffer; PBD: Perfusion buffer with dispase; PBC: Perfusion buffer with collagenase; WB: Washing buffer; WBD: Washing buffer with DNase.

Perfusion apparatus setup

To ensure flow of appropriate buffers, a peristaltic pump with adjustable speed was used in our perfusion system. This system was formed by mounting a peristaltic pump with two silicone tubings immersed in a variable-sized water tank to accommodate the liver tissues. The water bath temperature was adjusted and maintained at 37 °C for liver undergoing perfusion and digestion. The pump speed was set at constant flow.

Reagents and solutions preparation

The method used several reagents that had been prepared in advance (Table 2). The reagents were mixed thoroughly and filter solutions were prepared using 0.22-μm filters, after which the pH value was adjusted to 7.4 and the solutions were stored at 4 °C.

Hepatocyte isolation

We adopted a rigorous and stringent isolation protocol for all liver tissues. Precise time between tissue resection and isolation commencement was recorded.

Liver wedges were usually obtained from segments II/III and were carefully cut and weighed. Primary human hepatocytes were isolated under strict sterile conditions using a modified four-step retrograde perfusion technique (Figure 1). To eliminate interpersonal variability, all of the subsequent isolation procedures were carried out by single individual (Meng FY).

The liver samples were cannulated into the main blood vessel on the cut surface. The liver tissue was flushed with ice-cold perfusion buffer with EDTA (PBE) *via* blood vessels on the cut surface. This step was important to remove excess blood and help to determine the vessels that would offer optimal perfusion subsequently. The chosen vessel was cannulated with a suitable pipette tip, and flushed with PBE at 37 °C. In some cases, there might be more than one cut surface, or there might be a cut or tear on the outer capsule of the liver tissue (Glisson's capsule). These were required to be sewed-up in advance to ensure optimal perfusion of the tissue.

After flushing with perfusion buffer to clear the PBE,

Table 2 Reagents and solutions preparation (prepared for 80 g liver sample)

Resolution	Ingredient	Concentration (g/L)
PB (Perfusion buffer)	Double-distilled water (3000 mL)	
	NaCl (27 g)	9
	KCl (1.26 g)	0.42
	NaHCO ₃ (6.3 g)	2.1
	Glucose (2.7 g)	0.9
	Hepes (14.34 g)	4.78
PBE (Perfusion buffer with EDTA)	PB (1000 mL)	
	EDTA (0.37 g)	0.37
PBD (Perfusion buffer with dispase)	PB (500 mL)	
	Dispase II (Sigma) (4.2 g)	8.4
PBC (Perfusion buffer with collagenase)	PB (500 mL)	
	Collagenase IV (Sigma) (0.25 g)	0.5
	CaCl ₂ •2H ₂ O (0.275 g)	0.55
WB (Washing buffer)	Double-distilled water (2500 mL)	
	NaCl (17.5 g)	7
	KCl (1.15 g)	0.46
	CaCl ₂ •2H ₂ O (0.325 g)	0.13
	Hepes (5.95 g)	2.38
	Bovine serum albumin (2.5 g)	1.0
WBD (Washing buffer with DNase)	WB (Washing buffer) (1500 mL)	
	MgCl ₂ •6H ₂ O (0.15 g)	0.1
	MgSO ₄ •7H ₂ O (0.15 g)	0.1
	DNase I (Sigma) (0.15 g)	0.1

the tissue was then continuously perfused with a pre-warmed digestion buffer solution (perfusion buffer with dispase and perfusion buffer with collagenase). After sufficient digestion, the liver Glisson's capsule was mechanically disrupted by using an operating knife blade. The isolated hepatocytes were released into the medium by gentle shaking, leaving behind the connective tissue and any undigested material. The resultant hepatocyte suspension was divided equally into sterile centrifuge bottles. The suspension was then filtered through a 500- μ m nylon mesh and centrifuged at $50 \times g$ for 2 min at 4 °C. We regularly applied a 10-min cell incubation step by using wash buffer solution containing DNase I (WBD). Cell clumps were broken up and damaged cells were digested. The suspension was then filtered (75 s), and the resultant cells were harvested by low-speed centrifugation at $50 \times g$ for 75 s. This was followed by washes in cold wash buffer solution, filtration (60 s) and another centrifugation step ($50 \times g$, 75 s, 4 °C). Finally, the resultant hepatocyte clumps were resuspended in cold William's Medium E (Sigma). Hepatocyte yield and viability were immediately determined using the standard trypan blue exclusion technique after isolation/purification.

Hepatocyte culture

Culture medium was William's E Medium supplemented with 100 μ g/mL streptomycin, 100 mU/mL penicillin and 10% fetal bovine serum (FBS). Freshly isolated hepatocytes were seeded in culture flasks at a concentration of 4×10^5 to 5×10^5 /mL. The culture medium was changed every 24 h. The morphology of the cultured cells was assessed throughout the entire

culture period using light microscopy. Phase-contrast microscopy pictures were taken with a Nikon Diaphot inverted microscope.

RESULTS

The type of liver tissues used and the hepatocytes isolated are shown in Table 1. The liver tissue donors had intrahepatic duct calculi ($n = 7$) and liver hemangioma ($n = 17$). To improve cell availability, we investigated the influence of WIT and cold ischemia time and liver donor characteristics on the outcome of freshly isolated hepatocytes from surgically-resected liver tissues. All patients (15 males and 9 females) were negative for hepatitis C virus, hepatitis B virus surface antigen, and human immunodeficiency virus. All of the patients had normal liver function tests prior to surgery. The mean donor age was 50.7 years (range: 23-79 years). Blood group was O⁺ in 8 cases, A⁺ in 9, AB⁺ in 1, and B⁺ in 6 (Table 1). Data from our work revealed that patient sex, age and blood group had no correlation with cell yield and viability.

Liver wedges were prepared as shown in Figure 2. Representative images of isolated primary human hepatocytes, in culture for the first week, are shown in Figure 3. Similar morphological changes in the cultured cells were observed during the first week. The cells maintained normal morphology for at least 1 wk during culture in William's E Medium.

We processed and isolated human hepatocytes from 24 liver wedges. The WIT, *i.e.*, the interval between clamping and bathing in ice-cold solution, averaged 17.5 ± 8.8 min (range: 5-35 min) (Table 1). The cold ischemia time, *i.e.*, the interval between liver resection



Figure 2 Preparation of liver wedges. Only patients who had undergone left hemi-hepatectomies were deemed suitable for obtaining normal resected liver tissue from (A). Cut-off end of a suitable pipet tip to obtain an optimal size to match the vessel opening and cannulate the chosen vessel (B, C). Primary human hepatocytes must be isolated under stringent and rigorous sterile conditions (D).

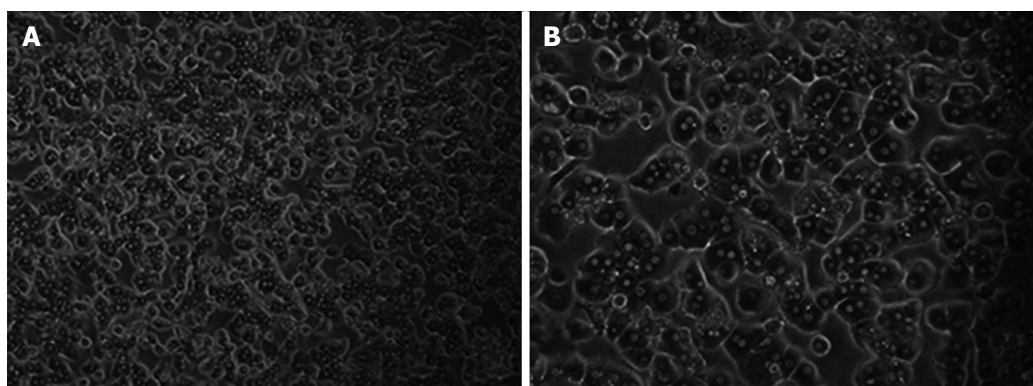


Figure 3 Phase-contrast photographs of primary human hepatocytes at 24 h after isolation. Magnification $\times 100$ (A) and $\times 200$ (B).

and perfusion, averaged 19.3 ± 3.3 min (range: 15-45 min). For the 7 samples of intrahepatic duct calculi, the method resulted in a hepatocyte yield of $3.49 \pm 2.31 \times 10^6$ hepatocytes/g liver, with $76.4\% \pm 10.7\%$ viability. However, for the 17 samples of liver hemangioma, we got better results for the hepatocyte yield ($5.4 \pm 1.71 \times 10^6$ cells/g vs $3.49 \pm 2.31 \times 10^6$ cells/g, $P < 0.05$) compared to the samples of intrahepatic duct calculi (Figure 4A). However, there seemed to be no clear difference in cell viability ($80.3\% \pm 9.67\%$ vs $76.4\% \pm 10.7\%$, $P > 0.05$) (Figure 4B). In our study, we obtained a cell yield of $5.31 \pm 1.87 \times 10^6$ hepatocytes/g liver when the samples weighed > 20 g. However, for the tissues that weighed ≤ 20 g, a reduction in yields

was found ($3.08 \pm 1.86 \times 10^6$ cells/g vs $5.31 \pm 1.87 \times 10^6$ cells/g, $P < 0.05$) (Figure 4C). In addition, no difference in cell viability was observed ($80.0\% \pm 9.85\%$ vs $76.0\% \pm 10.5\%$, $P > 0.05$) (Figure 4D).

All of the cultured primary hepatocytes demonstrated albumin synthesis in the first week (Table 3). Serum albumin concentrations were determined using immunonephelometry (Array; Beckman Instruments, Galway, Ireland). The isolated hepatocytes showed significantly increased albumin synthesis after 2 d of culture. When human hepatocytes were cultured in William's E Medium supplemented with 10% FBS, the cells maintained their polygonal shape until day 5 (Figure 3).

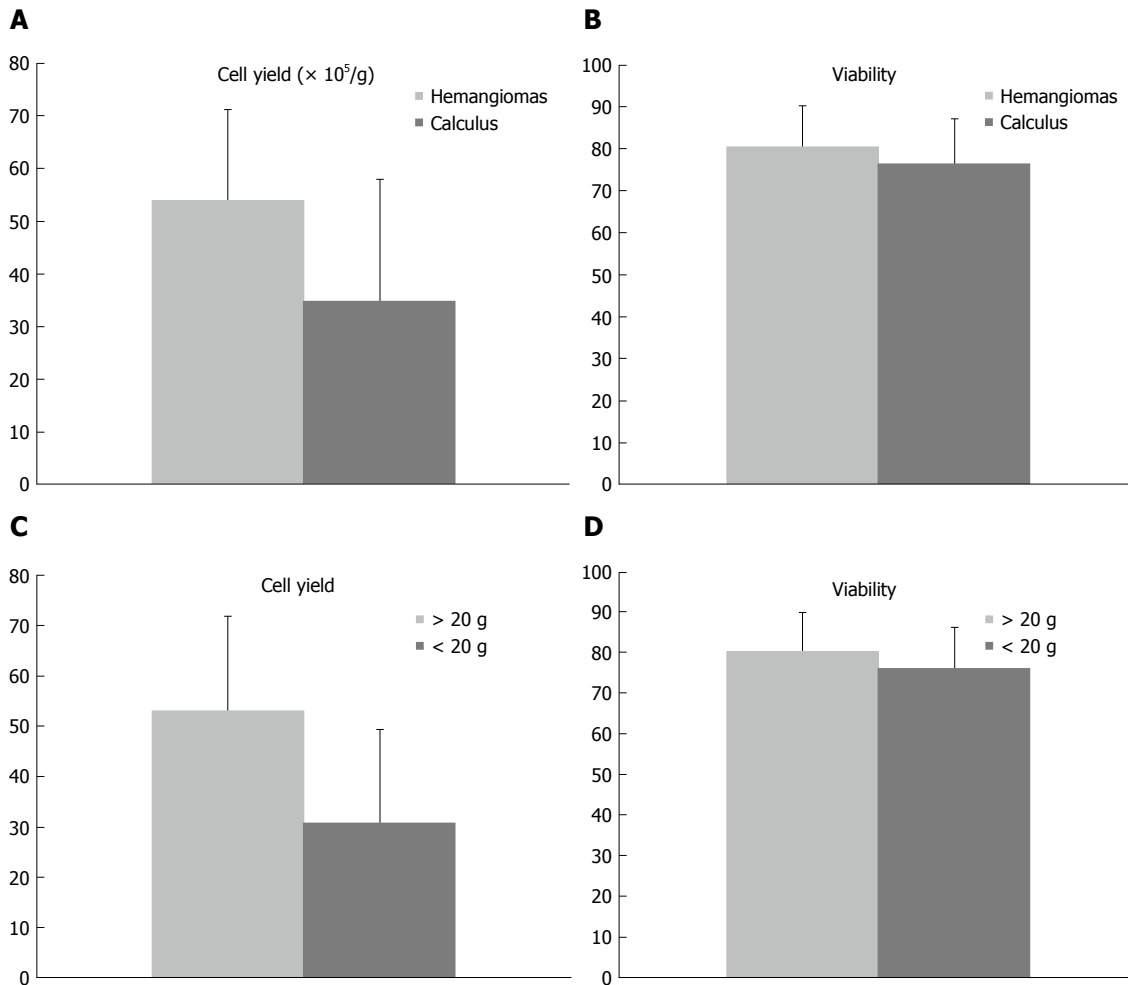


Figure 4 Samples of liver hemangioma can provide better results, in terms of cell yield, than intrahepatic duct calculi. In addition, the size of the tissues can affect the outcome of hepatocyte isolation.

Table 3 Concentrations of albumin in the cultured hepatocyte suspension

Time	Albumin (g/L)
24 h	0.79 \pm 0.31
7 d	1.36 \pm 0.42
10 d	1.09 \pm 0.21

The concentration of albumin in the William's E culture medium was about 0.5 g/L.

DISCUSSION

Hepatocyte isolation is a time-consuming and costly procedure^[12,13]. However, surgical specimens are of varying quality, which can affect the cell yield and viability. It is important to identify surgically-resected tissues with the best expectations concerning cell quality and yield^[14-16]. The aim of the present study was to analyze retrospectively the influence of the donor liver characteristics on the outcome of primary hepatocyte isolation from surgically-resected liver tissue. Once such a standardized method has been established, isolated hepatocytes will be easily accessible and more frequently available.

Hepatocyte isolation started in the mid-1960s^[17]. After that, many innovative techniques were introduced to improve the results^[8,18,19]. However, most of the innovative techniques have been applied exclusively to tissues obtained from resected liver tumors or from whole organ donors^[20]. We established a modified four-step collagenase retrograde perfusion technique for isolation of hepatocytes from non-diseased liver tissue removed at surgical resection.

The modified method, compared to the traditional method, can improve results, allowing isolation of a large number of hepatocytes of high quality. After hepatocyte isolation, a 10-min incubation step using DNase I (WBD) was used. Cell clumps break up and damaged cells are digested. Our technique resulted in a hepatocyte yield of $4.85 \pm 2.05 \times 10^6$ cells/g liver. The viability of the isolated hepatocytes, using the trypan blue exclusion technique, was $79.17\% \pm 9.90\%$.

Intrahepatic duct calculi and liver hemangioma are two common diseases among the local population. Tissues from patients undergoing partial hepatectomy are the most frequently available sources for hepatocyte isolation. However, not all of these tissues can be used to isolate large numbers of hepatocytes. Hepatic fibrosis

often occurs in patients with intrahepatic duct calculi, who generally do not support successful cell isolation.

Different with other experiences reported in the literature^[21], our results showed that the 17 samples of liver hemangioma had significantly higher yield of cells ($5.4 \pm 1.71 \times 10^6$ cells/g) than the samples of intrahepatic duct calculi ($3.49 \pm 2.31 \times 10^6$ cells/g, $P < 0.05$). However, there seemed to be no clear difference in cell viability ($80.3\% \pm 9.67\%$ vs $76.4\% \pm 10.7\%$, $P > 0.05$).

Cytotoxic bile acids accumulate in the hepatocytes during cholestasis, which is thought to induce hepatocyte necrosis and contribute to development of liver cirrhosis^[22,23]. This could explain the lower cell yield observed in hepatocytes isolated from cholestatic livers^[24-26]. In contrast, Iqbal *et al.*^[27] observed no significant difference in cell yield and viability in hepatocytes isolated from resected cirrhotic livers, as compared to non-cirrhotic livers. Further investigation should be made to evaluate the usage of resected cirrhotic livers for cell isolation.

Compared to resected livers from patients with intrahepatic duct calculi, we more frequently obtained a reliable source of normal liver tissue from patients with hemangioma. Furthermore, we found that left lateral sector segments were usually suitable to obtain normal resected tissues, with proportionate volumes, after which the lobular blood vessels can be easily exposed for catheterization. Of the total 24 samples, 19 were obtained from left hemi-hepatectomy. The fragments of hepatic tissue were cut from the periphery of the discarded material, surrounded by the hepatic capsule. It is important that the liver lobe should be incised with a single cut, and then the lobular blood vessels can be exposed for catheterization. We consider that the first perfusion step to drain off blood in the vessels is critical, because it can significantly affect the outcome of the subsequent collagenase digestion.

Among the general features of the patient, blood group, age and sex, did not have any influence on the yield or viability of the isolated human hepatocytes. In contrast, several investigations have described a decrease in hepatocyte viability or yield with an increase in patient age^[8,28]. This may have resulted from the distribution of diseases among the different age groups, which can significantly affect the outcome of hepatocyte isolation^[8]. Patients aged > 50 years had malignant diseases mainly. In our study, all of the specimens were obtained from patients with benign liver disease. Without the interfering factors, we observed that patient age had no correlation with cell yield and viability.

WIT can affect the outcome of hepatocyte isolation. It is reported that porta hepatis clamping during liver resection results in a low yield of isolated hepatocytes^[26]. There is a negative correlation between WIT (< 30 min) and low cell yield^[26]. It is also reported that intermittent clamping is more damaging to the liver than continuous clamping^[29]. As for cold ischemia time,

it is reported that ≤ 24 h has no effect on hepatocyte yield and viability^[28,30,31]. As shown in our study, when resected liver tissue is used for hepatocyte isolation, the WIT and cold ischemia time are always short. So, WIT and cold ischemia time seem to have no obvious effect on the outcome of hepatocyte isolation from surgically-resected tissues.

The size of the tissue can affect the outcome of hepatocyte isolation. Alexandre *et al.*^[26] concluded that the percentage of digested liver decreased when tissue weights were > 100 g. In our study, we found a reduction in yields when the tissue weighed ≤ 20 g (Table 2). One reason is that small tissue samples always have several cut surfaces, without an integrated hepatic capsule. Another reason is that it is difficult to find a visible vessel orifice on the cut surface to perfuse collagenase solutions. In contrast to the large tissue samples, small samples always have a longer isolation process with insufficient digestion. This suggests that the four-step perfusion technique for tissues ≤ 20 g should be modified.

One possible method to improve the isolation outcome of small tissue samples is to glue the section surfaces in order to avoid leakage of the perfusate. However, some studies have reported that the use of glue tends to decrease the yield of viable cells^[26]. Another possible method to increase the hepatocyte yield is to separate the viable cells using a Percoll centrifugation technique. We need to consider whether Percoll centrifugation is acceptable for the large quantity of hepatocytes required for clinical application. Due to the small numbers of tissues in our study, further investigations are needed to evaluate the optimal procedure for small resected specimens.

In conclusion, benign diseased livers appear to be a valuable source of a large number of isolated human hepatocytes. We consider that patient age has no correlation with cell yield and viability. Mild cirrhotic livers should not be arbitrarily excluded from cell isolation. We recommend, for optimal isolation, to use liver specimens weighing > 20 g and to avoid the use of liver specimens with severe fibrosis.

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COMMENTS

Background

There are still no systematic studies investigating resected specimens from patients with benign liver disease for large-scale hepatocyte isolation. Therefore, available data about the effect of the donor liver on isolated hepatocyte yield are scarce.

Research frontiers

The authors established an efficient technique for a special kind of liver

samples for large-scale human hepatocyte isolation. They retrospectively analyzed a 5-year experience of human hepatocyte isolation from surgically-resected normal tissues and evaluated the effect of donor liver characteristics on the hepatocyte isolation outcome.

Innovations and breakthroughs

The authors consider that patient age has no correlation with cell yield and viability. Mild cirrhotic livers should not be arbitrarily excluded from cell isolation. They recommend, for optimal isolation, to use liver specimens weighing > 20 g and to avoid the use of liver specimens with severe fibrosis.

Applications

Benign diseased livers appear to be a valuable source of a large number of isolated human hepatocytes.

Terminology

WIT (warm ischemia time), the interval between clamping and bathing in ice-cold solution. Cold ischemia time, the interval between storing in ice-cold solution and starting isolation.

Peer-review

The manuscript is well written. The study, conducted for 5 years, is well described and the results are clear and explained the target of the authors research in a good manner.

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Retrospective Cohort Study

Etiology of chronic liver diseases in the Northwest of Italy, 1998 through 2014

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Abstract

AIM

To assess the etiology of chronic liver diseases (CLD) from 1998 to 2014 at the outpatient clinic of Gastroenterology of the main hospital in Northwest of Italy among those dedicated to hepatology.

METHODS

A random sample of charts of patients referred to for increased liver enzymes between January 1998 and December 2006, and between January 2012 and

December 2014 were reviewed. Etiology search included testing for hepatitis B virus (HBV), hepatitis C virus (HCV), autoimmune hepatitis, primary biliary cirrhosis, Wilson's disease and hereditary hemochromatosis. A risky alcohol consumption was also considered. Non-alcoholic fatty liver disease (NAFLD) was diagnosed in patients with histological and/or ultrasound evidence of steatosis/steatohepatitis, and without other causes of CLD.

RESULTS

The number of patients included was 1163. Of them, 528 (45%) had positivity for HCV and 85 (7%) for HBV. Among the virus-free patients, 417 (36%) had metabolic disorders whereas the remaining had history of alcohol abuse, less prevalent causes of CLD or concomitant conditions. In comparison to 1998-2000 (41%), a reduction of HCV alone-related cases was detected during the periods 2001-2003 (35%, OR = 0.75, 95%CI: 0.53-1.06), 2004-2006 (33%, OR = 0.70, 95%CI: 0.50-0.97) and 2012-2014 (31%, OR = 0.64, 95%CI: 0.46-0.91). On the contrary, in comparison to 1998-2000 (31%), metabolic-alone disorders increased in the period 2004-2006 (39%, OR = 1.37, 95%CI: 0.99-1.91) and 2012-2014 (41%, OR = 1.53, 95%CI: 1.09-2.16). The other etiologies remained stable. The increase of incidence of metabolic-alone etiology during the period 2004-2006 and 2012-2014 tended to be higher in older patients (≥ 50 years) compared to younger ($P = 0.058$).

CONCLUSION

In the Northwest of Italy, during this study period, the prevalence of HCV infection decreased notably whereas that of NAFLD increased.

Key words: Chronic liver diseases; Cirrhosis; Hepatitis C virus; Hepatitis B virus; Nonalcoholic steatohepatitis

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Core tip: The epidemiological knowledge of variations of etiology of chronic liver diseases (CLD) is crucial for health policy of resource allocation and for planning strategies of prevention and treatment. Our study, carried out in a large population from 1998 to 2014 period, shows that, in Northwest Italy, viral CLD decreased and CLD due to metabolic disorders remarkably increased. These results suggest the need to perform a strategy of rigorous education and counseling, in particular in overweight and obese subjects.

Saracco GM, Evangelista A, Fagoonee S, Ciccone G, Bugianesi E, Caviglia GP, Abate ML, Rizzetto M, Pellicano R, Smedile A. Etiology of chronic liver diseases in the Northwest of Italy, 1998 through 2014. *World J Gastroenterol* 2016; 22(36): 8187-8193 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i36/8187.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i36.8187>

INTRODUCTION

The epidemiology of chronic liver diseases (CLD) in Italy is now changing due to the decreasing rate of viral hepatitis^[1] and the increasing new epidemic of a wide spectrum of metabolic disorders like steatosis, non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH)^[2]. The last three decades witnessed extensive diagnosis of the major hepatitis viruses, such as hepatitis C virus (HCV), hepatitis B virus (HBV) and hepatitis D virus (HDV) and their diffuse treatment with antiviral therapy. In addition, vaccination programs against HBV, which in Italy became mandatory since 1991, helped to achieve the complete immunization of newborn, teenagers and young adults^[3,4]. This active approach for HBV prevention was also effective against the risk of acquiring a superimposed HDV infection. As a result, Italy has become a country with a low (< 0.3%) endemicity for HBV infection, and HDV infection is almost vanishing^[3]. However, HCV infection alone, despite the lowest rate of infection occurring to date in young adults, remains at risk of spread among subjects with a lifestyle prone to high risk behaviours or undergoing invasive medical procedures.

Unfortunately, the amelioration of hygiene and social conditions, has been accompanied by a rapid change in eating habits of Italian people, with a diet richer in sugar and lipids compared to the past healthy Mediterranean regimen. Furthermore, overweight and obesity in children and adults have reached prevalence rate of 10%-15%^[5]. Such figures, although lower compared to those reported in North America^[6], are substantial. We are thus facing a significant change in the main causes of CLD compared to the recent past. Hence, the knowledge of the new etiological pattern of CLD has become very important in planning both the strategy of health policy and clinical recommendations for the next decades.

The Unit of Gastroenterology of Molinette Hospital represents the main facility in the Northwest of Italy (with a population of around 6 million people) among those dedicated to the management of liver diseases. Since, in this area, there are no updated data on the trend of the etiology of CLD, the aim of the present study was to address this issue over the period 1998-2014.

MATERIALS AND METHODS

A sample of charts of patients referred to by the general practitioners for hepatology reasons, from January 1998 to December 2014, was reviewed. In our outpatient facility, the medical personnel is organized in work teams that follow cohorts of patients with specific pathologies. The mean number of consultations is from 10000 to 12000 per year^[7]. Patients were divided into three main cohorts: (1) with suspected or known chronic liver disease; (2) with suspected or known bowel diseases; and (3) with suspected or known diseases of the upper

Table 1 Patients characteristics *n* (%)

Variable	<i>n</i> = 1163
Age, median (IQR)	52 (40-62)
Males	644 (55)
Etiology	
HCV	528 (45)
HCV alone	407 (35)
Metabolic	557 (48)
Metabolic alone	417 (36)
HBV	85 (7)
HBV alone	67 (6)
Period	
1998-2000	295 (25)
2001-2003	251 (22)
2004-2006	336 (29)
2012-2014	281 (24)

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

gastrointestinal tract. Consultations are usually assigned into "first" and "control" in order to check the number of incidental cases in comparison to prevalent cases. For the purpose of this study, only patients with the first consultation were included. We analyzed the triennial prevalence of different causes of liver disease during the periods 1998-2000, 2001-2003, 2004-2006 in order to detect early changes in etiological trends. The cohort 2012-2014 was included to confirm the previous trend also in recent years. Since in the period 2007-2012 the outpatient clinic changed location, part of the data have not been homogeneously collected, due to logistic problems. Hence, to avoid biases we excluded this period from the analysis.

The more prevalent indication for hepatology consultations was the increase in liver enzymes. The search for etiology included testing for HBV serological markers (hepatitis B surface antigen, antibodies to hepatitis B core antigen), HDV serological markers (antibodies to HDV, IgG and IgM), and HCV serological markers (antibodies to HCV). Viral replication was measured by testing serum HBV-DNA, HDV-RNA, or HCV-RNA, using quantitative assays mainly based on PCR techniques to amplify the target nucleic acid (*i.e.*, COBAS TaqMan HBV and COBAS TaqMan HCV, Roche Diagnostics). We also collected a lifetime drinking history. The threshold for a risky alcohol consumption was set at 40 g/d for men or 20 g/d for women.

Autoimmune chronic hepatitis and cholestatic liver diseases were diagnosed on the basis of international standard criteria^[8-11]. Organ- and non-organ-specific autoantibodies were tested at the dedicated laboratory: Anti-nuclear, anti-mitochondrial, anti-smooth muscle and anti-liver/kidney microsome type 1 autoantibodies. Autoantibodies were evaluated by indirect immunofluorescence on murine liver and kidney. IgA, IgG and IgM were detected with fluorescein isothiocyanate.

Hereditary hemochromatosis was diagnosed according to the presence of abnormal ferritin and saturated transferrin serum values, genetic test, or

liver histology. Wilson's disease was diagnosed on the basis of accepted international criteria^[12,13]. Patients without known causes of CLD but serum aminotransferases elevation, histological and/or ultrasound evidence of hepatic steatosis/steatohepatitis, were considered as suffering from NAFLD or NASH^[14].

The study conformed to the guidelines of the 1975 Declaration of Helsinki and was approved by the local Hospital ethical committee. Data were managed with respect of patients' privacy.

Statistical analyses

Since the patient records were not available in electronic format, assessments of the etiologies of the CLD were evaluated in a review of random samples from physical files. No formal power analysis was performed respect to a statistical comparison and the sample size was determined on the basis of resources available for in-depth review of the medical records. A mean sample of 900 medical records (in order to obtain approximately 100 patients per year) have been extracted according to an ordered sequence generated using a uniform distribution. For each patient with confirmed CLD, demographic, clinical, and etiological data were recorded. The change of (1) HCV; (2) HCV alone; (3) metabolic; and (4) metabolic alone, etiology during the analyzed periods (1998-2000, 2001-2003, 2004-2006, 2012-2014) was evaluated using logistic regression models, adjusting for age and sex. Potential effect modification by age (< 50, ≥ 50 years) was evaluated in the logistic model including an interaction between the variables age and period.

RESULTS

The features of the cohort included in the study are reported in Table 1. The overall number of enrolled patients was 1163 (644 males, 55%), with a median age of 52 (interquartile range: 40-62). Of this cohort, 528 (45%) had positivity for anti-HCV (35% HCV-alone) and 85 (7%) for HBV (6% HBV-alone). Among the virus-free patients, 417 (36%) had metabolic disorders. The remaining group included patients with history of alcohol abuse, less prevalent causes of CLD or concomitant conditions.

To gain insight into the temporal trend of the etiological pattern of CLD, four periods (1998-2000, 2001-2003, 2004-2006, 2012-2014) were analyzed. Focusing on the main etiologies, in comparison to 1998-2000 (41% of HCV alone), a reduction in HCV-related cases was detected during the periods 2001-2003 (35% of HCV alone, OR = 0.75, 95%CI: 0.53-1.06), 2004-2006 (33% of HCV alone, OR = 0.70, 95%CI: 0.50-0.97) and 2012-2014 (31%, OR = 0.64, 95%CI: 0.46-0.91) (Table 2). On the contrary, liver disease due to metabolic disorders increased from 31.2%, during the period 1998-2000, to 39% during the period 2004-2006 (OR = 1.37, 95%CI: 0.99-1.91)

Table 2 Hepatitis C virus Etiology by period

Period	Patients, <i>n</i>	HCV			HCV alone		
		<i>n</i> (%)	OR (95%CI)	<i>P</i> value	<i>n</i> (%)	OR (95%CI)	<i>P</i> value
1998-2000 (Ref.)	295	157 (53)	1	-	121 (41)	1	-
2001-2003	251	127 (51)	0.89 (0.63-1.24)	0.487	87 (35)	0.75 (0.53-1.06)	0.107
2004-2006	336	140 (42)	0.61 (0.45-0.84)	0.002	112 (33)	0.70 (0.50-0.97)	0.031
2012-2014	281	104 (37)	0.51 (0.36-0.71)	< 0.001	87 (31)	0.64 (0.46-0.91)	0.012

ORs were estimated by a logistic regression model adjusting for age and gender. HCV: Hepatitis C virus.

Table 3 Metabolic etiology by period

Period	Patients, <i>n</i>	Metabolic			Metabolic alone		
		<i>n</i> (%)	OR (95%CI)	<i>P</i> value	<i>n</i> (%)	OR (95%CI)	<i>P</i> value
1998-2000 (Ref.)	295	136 (46)	1	-	92 (31)	1	-
2001-2003	251	121 (48)	1.08 (0.77-1.51)	0.663	78 (31)	0.98 (0.68-1.41)	0.907
2004-2006	336	167 (50)	1.14 (0.83-1.56)	0.418	131 (39)	1.37 (0.99-1.91)	0.061
2012-2014	281	133 (47)	1.01 (0.73-1.41)	0.947	116 (41)	1.53 (1.09-2.16)	0.015

ORs were estimated by a logistic regression model adjusting for age and gender.

Table 4 Subgroup analysis hepatitis C virus etiology

Subgroup	Period	Patients, <i>n</i>	HCV (interaction <i>P</i> = 0.407)		HCV alone (interaction <i>P</i> = 0.252)	
			<i>n</i> (%)	OR (95%CI)	<i>n</i> (%)	OR (95%CI)
Age < 50 yr	1998-2000 (Ref.)	132	68 (52)	1	61 (46)	1
	2001-2003	114	53 (46)	0.80 (0.48-1.32)	35 (31)	0.51 (0.30-0.86)
	2004-2006	155	70 (45)	0.74 (0.46-1.18)	55 (35)	0.62 (0.38-1.00)
	2012-2014	111	45 (41)	0.55 (0.32-0.93)	37 (33)	0.52 (0.30-0.89)
Age ≥ 50 yr	1998-2000 (Ref.)	163	89 (55)	1	60 (37)	1
	2001-2003	137	74 (54)	0.96 (0.61-1.52)	52 (38)	1.03 (0.64-1.65)
	2004-2006	181	70 (39)	0.51 (0.33-0.79)	57 (31)	0.77 (0.49-1.20)
	2012-2014	170	59 (35)	0.46 (0.30-0.72)	50 (29)	0.75 (0.47-1.19)

ORs were estimated by a logistic model including an interaction between the variables age and period. HCV: Hepatitis C virus.

Table 5 Subgroup analysis metabolic etiology

Subgroup	Period	Patients, <i>n</i>	Metabolic (interaction <i>P</i> = 0.300)		Metabolic alone (interaction <i>P</i> = 0.058)	
			<i>n</i> (%)	OR (95%CI)	<i>n</i> (%)	OR (95%CI)
Age < 50 yr	1998-2000 (Ref.)	132	56 (42)	1	44 (33)	1
	2001-2003	114	54 (47)	1.21 (0.73-2.00)	41 (36)	1.10 (0.65-1.87)
	2004-2006	155	64 (41)	0.93 (0.58-1.49)	52 (34)	0.97 (0.59-1.59)
	2012-2014	111	52 (47)	1.08 (0.64-1.82)	44 (40)	1.16 (0.67-1.99)
Age ≥ 50 yr	1998-2000 (Ref.)	163	80 (49)	1	48 (29)	1
	2001-2003	137	67 (49)	0.98 (0.62-1.55)	37 (27)	0.87 (0.52-1.44)
	2004-2006	181	103 (57)	1.36 (0.89-2.08)	79 (44)	1.81 (1.15-2.83)
	2012-2014	170	81 (48)	0.97 (0.63-1.50)	72 (42)	1.86 (1.18-2.94)

ORs were estimated by a logistic model including an interaction between the variables age and period.

and 2012-2014 (41%, OR = 1.53, 95%CI: 1.09-2.16) (Table 3).

Stratifying by age groups, no significant differences were observed in CLD incidence due to HCV alone between younger patients (< 50 years) and older ones (≥ 50 years) (*P* = 0.252) (Table 4), whereas the incidence of metabolic causes alone during the period 2004-2006 and 2012-2014 in comparison to 1998-2000 tended to be higher in older patients (≥ 50

years) than in younger ones (from 29% to 44% and 42% in the former vs from 33% to 34% and 40% in the latter group; OR = 1.81 vs 0.97, OR = 1.86 vs 1.16, *P* = 0.058) (Table 5).

DISCUSSION

The asymptomatic nature of mild CLD impedes the accurate determination of its epidemiology. Hence,

it is crucial, periodically, to perform studies aiming at defining detailed incidence and prevalence of liver diseases as well as their etiology.

The findings of our study show that in Northwest Italy, the incidence and prevalence of HCV infection decreased notably. This feature is supported by studies reporting a sharp decline in viral infections in hepatology settings more in Northern than in Central and Southern Italy. In a multicentre study including more than 6000 inpatients and outpatients, admitted for increase in liver enzymes in the year 2001, 62.6% had HCV alone, 9.2% HBV alone and about 13% non-viral causes. With regard to Northern Italy, HCV alone was reported in the 33.2% of cases, equivalent to our 33.3%, while NAFLD was diagnosed in the 34.2% of cases^[1], similarly to 39% of our study in the period 2004-2006. Hence, the findings of our facility, that was not involved in the above reported study, independently confirm the changes in epidemiology of CLD of the same area. Quite different is the situation in Southern Italy, where results of the SCIROCCO Study Group for Liver Disease, have found that 62.9% of cases of CLD were HCV positive, 11.9% were HBV positive, 1.3% were HBV/HCV co-infected, 11.6% had an alcoholic liver disease and 10.1% had NAFLD^[15]. Thus, in the South of Italy, viral infection still remains the most common etiology and the main cause for referral to centers dedicated to hepatology. These findings are similar to those obtained in a US population-based study, performed in Connecticut (New Haven County), Oregon (Multnomah) and California (Oakland) in the period 1999-2001, where HCV alone (42%) or in combination with alcoholic liver disease (22%) were the principal causes of CLD, followed by NASH for 9% and HBV for 3%^[16].

Considering the general population, in Northern Italy, the Dionysos study, collecting data of 6917 inhabitants of 2 towns (Campogalliano and Cormons), reported that the overall prevalence of anti-HCV-positivity was 3.2%. When classified by age, the prevalence was rather low (< 1%) in subjects younger than 40 years but it raised markedly in subjects older than 60 years reaching a value of 10%^[17]. According to some studies, this trend is in keeping with the so-called "cohort-effect"^[18]. For instance, La Torre *et al.*^[18] estimated the HCV infection trends in Italy during the years 1996-2006. A strong reduction in HCV infection was observed (-12.45%), with no differences according to gender (-12.23% in males and females -12.8% in females). When stratified by age, the incidence rate decreased significantly, from 2.02 (age < 65 years) to 0.55 (age > 65 years) per 100000, without differences among groups. Moreover, with the new treatments based on direct antiviral agents (DAAs), that directly inhibit HCV replication targeting different viral-encoded proteins, a further drop in the prevalence of HCV can be expected in the next years^[19]. Currently, since the DAA treatment for HCV has been introduced in our

region in January 2015, an impact in the reduction of HCV prevalence found in our cohort could be excluded. Whether immigration is affecting the hepatitis virus epidemiology in Italy remains to be clarified.

Tightly linked to obesity and metabolic syndrome, NAFLD is emerging as potentially the overriding liver disease of the near future^[20]. In our study there was an increase in rate of diagnosis of NAFLD. Although the latter was present in all cohorts, it was significantly higher in older than in younger subjects. Considering that an Italian multicentre cross-sectional study has shown that 23% of cases of hepatocellular carcinoma (HCC) occurred in virus-free patients with an increasing prevalence in the elderly, the issue of the age needs to be highlighted. Surveillance resulted an independent predictor for either single HCCs less than 2 cm or HCCs meeting the Milan criteria detection^[21]. Moreover, in a cohort of US veterans, NAFLD and metabolic syndrome resulted the principal risk factors of 13% of patients with HCC without signs of cirrhosis^[22]. Thus, the strict surveillance of these type of patients, in particular in elderly, is mandatory. This is more relevant considering that almost half of NAFLD patients have normal liver enzyme levels^[2].

As part of the prevention program, an important role is played by behavior therapy for NAFLD. This is supported by previous observational studies^[23,24] and finally by a more recent randomized controlled trial^[25]. In the near future, therapeutic and behavior interventions might be supported by new technologies, such as smartphone applications and web-based platforms in order to permit an interactive engagement between patients and physicians^[26].

In contrast with several studies conducted in general populations or in specific cohorts (for example blood donors) with short period analysis, our study evaluated the incidence of the main etiologies in a hepatology setting during a long period. This could be important for health policy of resource allocation. The health sector is characterized by vast demand and a lack of funds due to budget constraints. Prioritization is therefore necessary. Updating disease burden data is thus crucial in developing policy and prevention strategies, prioritizing research and appropriately allocating resources.

Some limitations of the present study have to be recognized. Estimates resulting from our outpatient clinic does not represent the general population. Nevertheless, data sources were critically assessed to avoid under- or over-estimation and to make sure that the results were consistent with general Italian population epidemiology^[18]. The potential heterogeneity in collecting data is limited both by the fact that, in our outpatient clinic^[7], all authors follow International Guidelines and that data were not collected in an outpatient clinic dedicated to a specific etiology (for example, exclusive for NASH or for viral hepatitis) but in a general hepatology one. As the primary aim

of our study was the definition of the epidemiologic pattern of these diseases over time, no data regarding CLD stages are provided. Another limitation is the lack of data in the period 2007-2012, due to the logistic problems above described.

In conclusion, our study carried out in a large population shows that, in Northwest Italy, there is a trend towards a decline in viral CLD and a relevant increase in liver diseases due to metabolic disorders. These results suggest the need for a strategy of rigorous counseling in the whole population, and particularly, in subjects with metabolic disorders.

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COMMENTS

Background

The epidemiology of chronic liver diseases (CLD) in Italy is now changing due to the decreasing rate of viral hepatitis and the increasing new epidemic of a wide spectrum of metabolic disorders like steatosis, non-alcoholic fatty liver disease and steatohepatitis. Since, in North-West Italy, there are no updated data on the trend of the etiology of CLD, the aim of the present study was to address this issue over the period 1998-2014.

Research frontiers

The epidemiological knowledge of variations of etiology of CLD is crucial for health policy of resource allocation and for planning strategies of prevention and treatment.

Innovations and breakthroughs

The present study included 1163 patients with CLD. Of them, 528 (45%) had positivity for hepatitis C virus (HCV) and 85 (7%) for hepatitis B virus. Among the virus-free patients, 417 (36%) had metabolic disorders whereas the remaining had history of alcohol abuse, less prevalent causes of CLD or concomitant conditions. In comparison to 1998-2000 (41%), a reduction of HCV alone-related cases was detected during the periods 2001-2003 (35%, OR = 0.75, 95%CI: 0.53-1.06), 2004-2006 (33%, OR = 0.70, 95%CI: 0.50-0.97) and 2012-2014 (31%, OR = 0.64, 95%CI: 0.46-0.91). On the contrary, in comparison to 1998-2000 (31%), metabolic-alone disorders increased in the period 2004-2006 (39%, OR = 1.37, 95%CI: 0.99-1.91) and 2012-2014 (41%, OR = 1.53, 95%CI: 1.09-2.16).

Applications

The study presented, carried out in a large population from 1998 to 2014 period, shows that, in Northwest Italy, viral CLD decreased and CLD due to metabolic disorders remarkably increased. These results suggest the need to perform a strategy of rigorous education and counseling, in particular in overweight and obese subjects.

Peer-review

In this study, the authors' give a brief overview of the epidemiology of chronic liver disease in their region over a 16 year period. However, there are several important methodological flaws, the most obvious of which is the lack of data between the years 2007-2010. This should undoubtedly be addressed by the authors before their manuscript is considered for publication. Furthermore, there also exists the question of the relevance of these data. The authors present a limited vision of the epidemiological trends, with no additional data such as concomitant diseases, obesity rates, antiviral treatment received, etc.

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Retrospective Study

Development of a prognostic scoring system for resectable hepatocellular carcinoma

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Abstract

AIM

To develop a prognostic scoring system for overall survival (OS) of patients undergoing liver resection (LR) for hepatocellular carcinoma (HCC).

METHODS

Consecutive patients who underwent curative LR for HCC between 2000 and 2013 were identified. The series was randomly divided into a training and a validation set. A multivariable Cox model for OS was fitted to the training set. The beta coefficients derived from the Cox model were used to define a prognostic scoring system for OS. The survival stratification was then tested, and the prognostic scoring system was compared with

the European Association for the Study of the Liver (EASL)/American Association for the Study of Liver Diseases (AASLD) surgical criteria by means of Harrell's C statistics.

RESULTS

A total of 917 patients were considered. Five variables independently correlated with post-LR survival: Model for End-stage Liver Disease score, hepatitis C virus infection, number of nodules, largest diameter and vascular invasion. Three risk classes were identified, and OS for the three risk classes was significantly different both in the training ($P < 0.0001$) and the validation set ($P = 0.0002$). Overall, 69.4% of patients were in the low-risk class, whereas only 37.8% were eligible to surgery according to EASL/AASLD. Survival of patients in the low-risk class was not significantly different compared with surgical indication for EASL/AASLD guidelines (77.2 mo *vs* 82.5 mo respectively, $P = 0.22$). Comparison of Harrell's C statistics revealed no significant difference in predictive power between the two systems (-0.00999 , $P = 0.667$).

CONCLUSION

This study established a new prognostic scoring system that may stratify HCC patients suitable for surgery, expanding surgical eligibility with respect to EASL/AASLD criteria with no harm on survival.

Key words: Hepatocellular carcinoma; Liver resection; Liver cirrhosis; Prognosis; Survival study

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Core tip: European Association for the Study of the Liver (EASL)/American Association for the Study of Liver Diseases (AASLD) guidelines recommend liver resection (LR) for hepatocellular carcinoma (HCC) only for single nodules of any size in patients without tumor related symptoms, no clinically significant portal hypertension and normal bilirubin. In this study we investigated the prognostic factors for survival of patients who underwent LR for HCC. We built a prognostic scoring system to stratify post-resection prognosis, and we identified a larger subset of patients with an expected survival that equates that of patients undergoing LR according to guidelines. Thus, the current EASL/AASLD indications for LR can be safely expanded, with no detrimental effect on patients' prognosis.

Sposito C, Di Sandro S, Brunero F, Buscemi V, Battiston C, Lauterio A, Bongini M, De Carlis L, Mazzaferro V. Development of a prognostic scoring system for resectable hepatocellular carcinoma. *World J Gastroenterol* 2016; 22(36): 8194-8202 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i36/8194.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i36.8194>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary tumor of the liver, and it is the third cause of cancer death worldwide^[1]. Most HCC cases (from 65% to 90%) occur in the context of chronic hepatitis and cirrhosis^[2], which are attributable mainly to chronic hepatitis B virus or hepatitis C virus (HCV) infections, followed by chronic alcohol abuse, obesity and diabetes^[3]. The estimated rate of each of these risk factors varies depending on the different regions of the world.

The prognosis of patients with HCC and the choice among the available therapeutic options, largely depends on tumor extension and underlying liver function. According to the European Association for the Study of the Liver (EASL)^[4] and the American Association for the Study of Liver Diseases (AASLD)^[5] guidelines, treatment allocation is routed by the Barcelona Clinic Liver Cancer staging system (BCLC)^[6]. In particular liver resection (LR) is considered as the first-line treatment only for patients at an early stage of the disease, namely those with an optimal liver function (Child-Pugh A, normal bilirubin and absence of clinically relevant portal hypertension), a preserved physical condition (ECOG Performance Status of 0), and a single tumor nodule with no evidence of extra-hepatic spread nor involvement of major vascular structures. In this subset of optimal patients, a 5-year overall survival (OS) of approximately 70% may be expected, similar to that of liver transplantation^[7]. Several field practice studies have ascertained that LR is often offered outside these conventional indications, and various authors reported acceptable survival rates for patients with HCC resected at a more advanced stage because of macrovascular invasion^[8,9], multiple nodules or impaired liver function^[10,11]. In addition, more recent studies demonstrate a survival benefit of radical surgery with respect to the available treatment alternatives across the different BCLC stages^[12-14].

The objective of this study is to investigate the prognostic factors for survival of patients who underwent LR for HCC at two referral centers - in which the surgical indication was not restricted to the current guidelines - and to build a prognostic scoring system to stratify post-treatment prognosis and possibly to expand the actual western indications to LR without harmful adverse outcomes.

MATERIALS AND METHODS

From January 2000 to March 2013 data from all patients who underwent a curative LR for HCC at the Departments of Gastrointestinal Surgery and Liver Transplantation of the Istituto Nazionale dei Tumori and the Ospedale Niguarda Ca' Granda of Milan were prospectively collected and entered in a master database. Patients with extrahepatic disease at

diagnosis and patients who were censored within two months were excluded from the present study. The master database contained 138 variables, including demographic, clinical, laboratory, treatment and survival data of each patient. The data were retrieved from the database for the purpose of this study after approval from the local institutional review boards.

Criteria for diagnosis and indication for LR

Criteria for HCC diagnosis were in accordance with EASL/AASLD guidelines evolution^[4,5,15]. The diagnosis of HCC was made on sequential contrast-enhanced imaging studies [chest computed tomography (CT), magnetic resonance imaging (MRI), or ultrasound] unless one study conclusively demonstrated a tumor with arterial enhancement and venous washout. In cases lacking conclusive radiological diagnosis, ultrasound-guided biopsy was used in both centers.

LR was performed within conventional guidelines but also beyond EASL/AASLD recommendations in all patients in whom surgical tumor removal was possible with a risk/benefit ratio in favor of surgical indication when compared with other available options such as liver transplantation, loco-regional therapies (ablation, transarterial chemoembolization or radioembolization) or systemic therapies (Sorafenib). Indication for surgery was always discussed in a multidisciplinary HCC board with hepatologists, oncologists, radiologists and surgeons.

Preoperative workup and definitions

No neoadjuvant locoregional/systemic treatments were indicated before surgery. Chest CT scan and contrast-enhanced abdominal CT scan or MRI were used for preoperative staging and volume assessment. The day before surgery, a thorough physical examination was accomplished, together with a complete biochemistry panel including serum alpha-fetoprotein (AFP) levels, and an indocyanine green retention test at 15 min. Liver function and reserve were determined according to the Child-Turcotte-Pugh (CTP) and Model for End-stage Liver Disease (MELD) scores. Presence of clinically relevant portal hypertension was defined as the presence of esophageal or gastric varices detectable at endoscopy or splenomegaly (major diameter > 12 cm) with a platelet count < 100000/mm³^[5]. Minor hepatectomy was defined as the resection of ≤ 2 adjacent liver segments^[16].

Perioperative management

All patients received low-molecular weight heparin the day before surgery and 2 g of cefazolin 30 min before skin incision. After accessing the peritoneal cavity, patients underwent complete abdominal exploration. Intra-operative ultrasound was used to assess tumor characteristics, exclude the presence of adjunctive focal lesions in the liver, ascertain intrahepatic vascular and biliary anatomy, individualize the resection plane with

a tumor-free margin of at least 1 cm and eventually decide on resectability. Anatomical resection was always attempted although the final decision on it was strictly dependent on the patient's tumor and liver conditions. Surgery was always performed within a fluid minimization protocol, particularly during hepatic dissection; a central venous pressure lower than 5 mm/Hg was targeted.

Follow-up schedule

After hospital discharge, patient follow-up was performed in a dedicated liver cancer clinic with hepatological and surgical competences in place to treat the underlying liver diseases and detect possible recurrence of HCC. Physical examination, biochemistry with AFP level measurement, chest CT scan and contrast-enhanced abdominal CT scan or MRI were performed every 4 mo for the first two years and every 6 mo thereafter. Anti-cancer treatment was not applied until recurrence. When recurrence was noted, each patient was treated according to disease presentation.

Statistical analysis

Categorical variables were reported as the number of cases and percentage; continuous variables were expressed as median and interquartile range (IQR). OS was estimated by the Kaplan-Meier method and calculated from the time from the date of hepatic resection to the earliest of death or last follow-up evaluation. For patients who underwent liver transplant (LT) either for HCC recurrence or end-stage liver disease, survival was censored the day before LT.

All eligible patients were randomly allocated into a training set or a test set in an approximately 1:1 ratio with seed set (16438) to make the procedure reproducible. For all subjects an independent uniform variable was generated and rounded to the closest integer: Two groups were identified according to the 0/1 result. The characteristics of patients in the training and test sets were compared using the Pearson chi-square test (or Fisher exact test, if necessary) for categorical variables, the Student *t*-test for continuous variables and the log-rank test for time-to-event data.

In the training set a Cox proportional hazards regression model was used to identify the baseline preoperative characteristics predicting OS, and those variables identified as significant in the univariate analysis at the level of $P < 0.05$ were tested in the multivariable setting. The proportionality assumption was verified by Schoenfeld residual analysis. A prognostic score was then derived using the independent variables weighed according to the estimated β regression coefficient of the final Cox model. The risk estimate associated with each point was then calculated using the Cox proportional hazards model according to the formula:

$$\hat{p} = 1 - S_0(t) \exp(\sum_{i=1}^p \beta_i X_i - \sum_{i=1}^p \beta_i \bar{X}_i)$$

Three prognostic stages (low risk, medium risk

Table 1 Baseline patients' characteristics and comparison between the training and validation sets *n* (%)

	Training set (<i>n</i> = 480)	Validation set (<i>n</i> = 437)	<i>P</i> value
Age (yr)	67 (61-73)	68 (61-73)	0.684
Gender, male	374 (77.9)	331 (75.7)	0.436
ECOG PS			0.362
0	452 (94.2)	405 (92.7)	
1-2	28 (5.8)	32 (7.3)	
Child-Pugh			0.118
A	453 (94.4)	401 (91.8)	
B	27 (5.6)	36 (8.2)	
MELD score	8 (7-10)	8 (7-10)	0.791
Etiology			0.516
Cryptogenic	104 (21.7)	88 (20.1)	
HBV only	94 (19.6)	71 (16.2)	
HCV only	222 (46.25)	217 (49.7)	
Alcohol	48 (10.0)	45 (10.3)	
HBV + HCV	12 (2.5)	16 (3.7)	
HCV infection	247 (51.5)	246 (56.3)	0.142
Portal hypertension	165 (34.4)	155 (35.5)	0.728
Platelet count (10 ³ /μL)	157 (25-505)	154 (26-914)	0.779
AFP, ng/mL (<i>n</i> = 663)	14.3 (4.7-121.5)	11.4 (4.3-71)	0.160
ICG-R15 (<i>n</i> = 400)	15 (6.1-25)	16 (7.7-22.3)	0.424
Total bilirubin (≥ 1.2 mg/dL)	148 (30.8)	126 (28.8)	0.509
Number of lesions (> 3)	10 (2.1)	10 (2.3)	0.832
Largest diameter (> 5 cm)	143 (29.8)	124 (28.4)	0.637
Portal invasion	22 (4.6)	14 (3.2)	0.283
Extent of hepatectomy (major)	85 (17.7)	78 (17.8)	0.956
Follow-up status (dead)	240 (50.0)	202 (46.2)	0.253
Follow-up time (mo)	35.9 (16.3-61.0)	32.5 (16.7-55.8)	0.254
Overall survival	59.3 (50.2-66.6)	56.4 (47.0-75.8)	0.833

Continuous variables are reported as median values and interquartile range, categorical variables as the number of cases and percentage. Patients' characteristics in the two sets are compared using the Pearson χ^2 test (or Fisher exact test, if necessary) for categorical variables, the Student *t*-test for continuous variables and the log-rank test for time-to-event data. ECOG PS: Eastern Cooperative Oncology Group Performance Status; MELD: Model for End-stage Liver Disease; AFP: α -fetoprotein; ICG-R15: Indocyanine green retention test at 15 min.

and high risk of death at 5 years from surgery) were identified according to the changes in the risk estimates for each point increase of the score. OS curves in the training and test sets for the three prognostic stages were obtained with the Kaplan-Meier method and compared by means of log-rank test.

Patients in the "low-risk" category were considered as "optimal candidates for surgery" according to the model's predicted survival; the dichotomization of the model (optimal vs non-optimal candidate for surgery) was then compared with the EASL/AASLD indications through calculation of Akaike Information Criteria^[17], comparison of Harrell's C statistics^[18] and comparison of survival rates at 5 years.

All analyses were 2-tailed and the threshold of significance was assessed at *P* < 0.05. The statistical analysis was performed using STATA®, version 13 (StataCorp LP, United States). The statistical methods of this study were reviewed by Dr. Federica Brunero, Clinical Trial Office and Biomedical Statistic, Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy.

RESULTS

A total of 917 eligible adult HCC patients were included. Overall, the median age at presentation was 67 years

(IQR: 61-73 years). The majority of patients were men (705 subjects, 76.9%) and were predominantly classified as "fully active" (ECOG PS 0, 93.5%). Eight-hundred fifty-three patients (93.1%) had CTP grade A liver function, and 616 patients (72.1%) had MELD score less than or equal to 9^[19]. In the majority of cases (46.2%), HCV was the etiology of liver disease and 320 patients (35%) had clinically relevant portal hypertension. Two-hundred sixty-seven patients (29.1%) had a tumor size greater than 5 cm and 897 subjects (97.8%) had up to three tumor nodules. Portal invasion was detected in 36 patients (3.9%). Thirty- and ninety-day mortality rates were 1.1% and 3.5%. Median follow-up of the entire series was 58.1 mo (95%CI: 52.3-63.9). During follow-up 442 deaths were registered. Survival at 5 and 10 years and median survival were 49.3%, 26.2% and 58.7 mo (95%CI: 51.5-65.9) respectively. Recurrence-free survival (RFS) at 3 and 5 years and median RFS were 43.7%, 31.8% and 28.8 mo (95%CI: 25.0-35.6) respectively.

Among the 917 patients, 480 (52.34%) and 437 (47.66%) were assigned randomly to the training set and the validation set, respectively. The demographic, clinical and laboratory characteristics of patients assigned in the two sets are presented in Table 1. Overall, the patients in the training and the validation sets shared

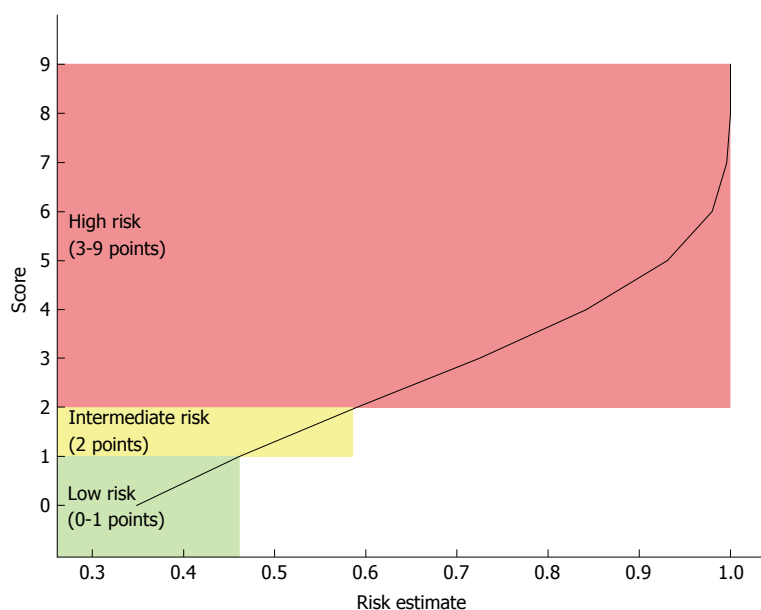


Figure 1 Scoring system according to risk estimates of death at 5-yr. Patients are considered at low risk with a score = 0-1 (risk estimates: 0.347-0.459), intermediate risk with a score = 2 (risk estimate: 0.59), and high risk with a score = 3-9 (risk estimates: 0.723-1).

Table 2 Multivariable Cox proportional hazards regression model (training set) and relative point system

Variable	HR	95%CI	P value	β	Points
MELD score ≤ 9					0
MELD score > 9	1.444	(1.080-1.931)	0.013	0.3674	1
HCV infection absent					0
HCV infection present	1.468	(1.112-1.937)	0.007	0.3839	1
Number of lesions ≤ 3					0
Number of lesions > 3	3.253	(1.434-7.380)	0.005	1.1795	3
Largest diameter ≤ 5 cm					0
Largest diameter > 5 cm	1.459	(1.085-1.963)	0.012	0.3779	1
Portal invasion absent					0
Portal invasion present	3.500	(2.016-6.073)	< 0.001	1.2526	3

MELD: End-stage Liver Disease; HCV: Hepatitis C virus.

similar characteristics, including survival and censoring pattern. Greater than 50% of patients presented with an active HCV infection. Because HCV infection was the prevalent aetiology of cirrhosis, we chose to compare it with all the other aetiologies grouped.

Development of the prognostic classification score

Results of the univariate analysis on preoperative characteristics are presented in Supplementary Table 1. Those preoperative variables identified as significant in the univariate analysis at the level of $P < 0.05$ were fitted in a multivariable Cox proportional hazards regression model within the training set. The proportionality of hazard ratios for all levels of all prognostic factors was verified. The beta coefficients were transformed into relative points and a point system was constructed according to the method described by Sullivan *et al.*^[20] (Table 2).

The total score ranged between 0 and 9. The risk estimates were calculated for each score using the Cox

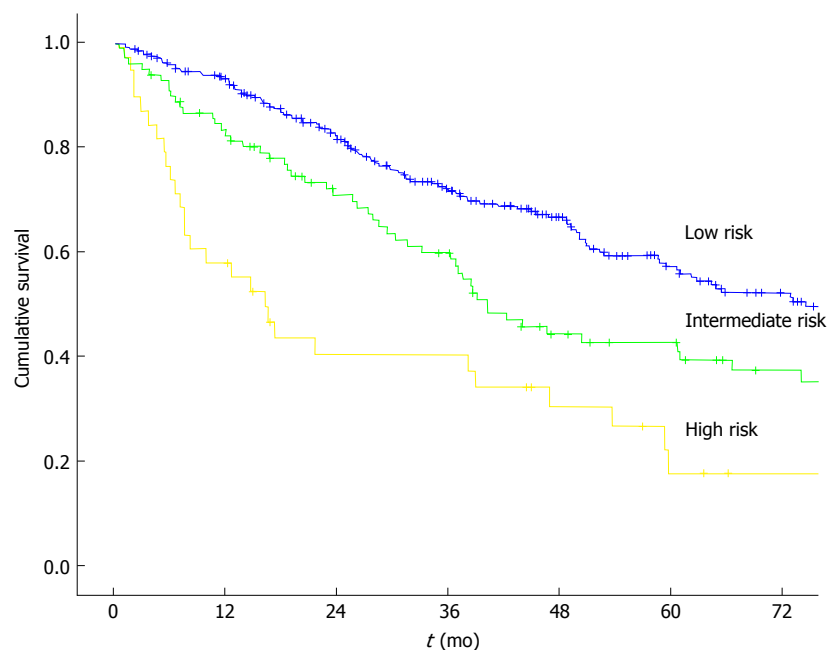
proportional hazards model, and three risk stages were defined according to changes in the risk estimates for each point increase (Figure 1): Low risk: 0 to 1 points; Intermediate risk: 2 points; High risk: 3 to 9 points.

OS curves for the three prognostic stages are presented in Figure 2. In the training set, a significant difference in survival between the three stages was demonstrated ($\chi^2 = 33.56$ and $P < 0.000$), and this finding was confirmed in the validation set ($\chi^2 = 23.67$ and $P = 0.0002$). When considering the case series as a whole, 5-year, 10-year and median survival were 57.2%, 31.2% and 77.2 mo (95%CI: 67.4-87.0) respectively in the low risk category, 40.3% 22.6% and 41.7 mo (95%CI: 34.7-48.7) respectively in the intermediate category and 22.3% 13.4% and 17.4 mo (95%CI: 10.1-24.6) respectively in the high risk category ($P < 0.000$). Three-year, 5-year and median RFS were 46.4%, 33.8% and 31.5 mo (95%CI: 25.3-35.7) respectively in the low risk category, 40.1% 28.1% and 29.9 mo (95%CI: 25.6-34.2) respectively in the intermediate category and 34.5% 25.9% and 12.5 mo (95%CI: 2.8-22.2) respectively in the high risk category ($P = 0.020$). Details on sites of HCC recurrence and treatments for recurrence are shown in supplementary Table 2.

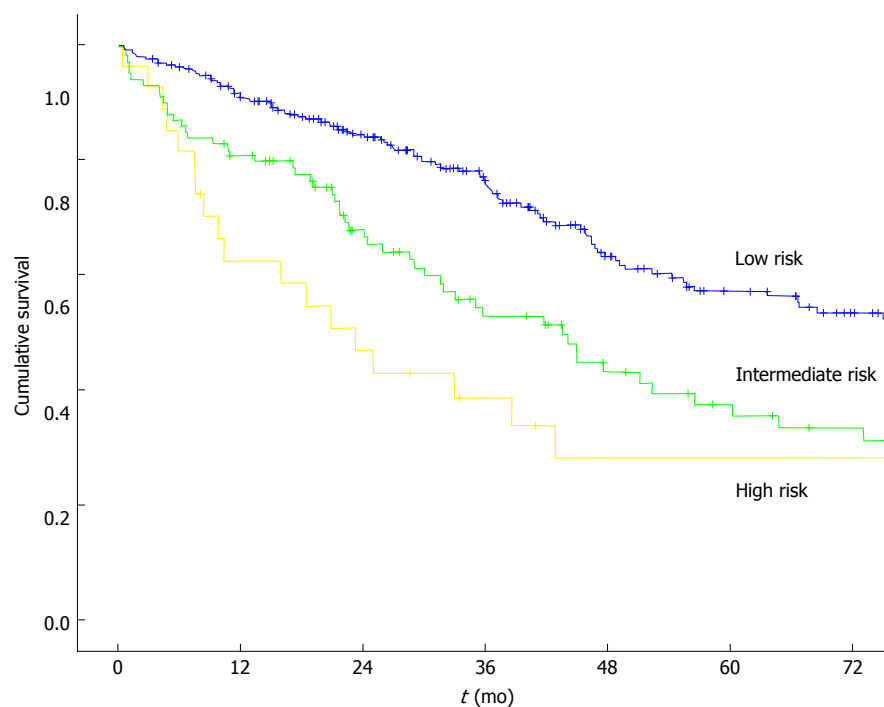
Identification of the ideal candidates for surgery and comparison with the EASL/AASLD guidelines

Patients in the low-risk category were considered as ideal candidates for LR according to the predicted survival. This criterion allowed the inclusion of 314 patients considered non-ideal candidates according to EASL/AASLD guidelines.

Overall, 593 patients (69.4% of the total of 854 evaluable patients according to both classifications) were ideal candidates for LR according to the proposed

A

Number at risk							
Low risk	309	270	205	155	114	81	62
Intermediate risk	97	78	58	48	30	26	17
High risk	38	22	13	13	8	4	2

B

Number at risk							
Low risk	284	239	188	135	89	66	51
Intermediate risk	99	78	52	36	25	19	15
High risk	27	16	12	8	5	5	3

Figure 2 Kaplan-Meier survival estimates for the risk classes in the training set (A) and the test set (B).

Table 3 Median overall survival and 1- 3- and 5-year survival probabilities for ideal and non-ideal candidates for liver resection according to European Association for the Study of the Liver/American Association for the Study of Liver Diseases and the current study

		No. Of patients (%)	Median OS (95%CI)	5-yr OS	10-yr OS
EASL/AASLD	Ideal	323 (37.8)	83 (73-108)	64.4	37.0
	Non-ideal	531 (62.2)	46 (41-52)	42.0	21.2
Current study	Ideal	593 (69.4)	77 (64-44)	57.2	31.2
	Non-ideal	261 (30.6)	38 (30-44)	35.8	20.0

EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases.

Milan score, whereas only 323 patients were ideal candidates for LR according to the EASL/AASLD guidelines (37.8% of the total). This finding resulted in a net increase of 31.6% of patients with ideal indication for LR.

Comparison with the EASL/AASLD surgical guidelines was performed by means of AIC, Harrell's C statistics and 5-year survival rates. AIC for EASL/AASLD surgical guidelines was 5323.259 and AIC for the Milan score was 4683.745. Harrell's C was 0.5971 and 0.5849 for the EASL/AASLD surgical guidelines and the proposed criteria respectively ($P = 0.617$), showing that there is no evidence that the two systems have different predictive power.

The 5-year survival rates for patients who are ideal candidates for surgery according to the two systems were not significantly different ($z = -1.6022$, $P = 0.06$), and median survivals did not differ ($z = -0.789$, $P = 0.22$) (Table 3).

DISCUSSION

LR still represents the cornerstone for any curability attempt in patients with HCC. A large burden of surgical literature has challenged the current Western guidelines. However, LR for HCC is recommended only for single nodules of any size in patients without tumor related symptoms, no clinically significant portal hypertension and normal bilirubin^[4,5]. If this profile is not fulfilled, postoperative morbidity may increase, and long-term survival may be significantly reduced. In contrast, when patients meet these criteria, long-term survival may equate that of LT. Thus, LR under restricted conditions maintains its role as a first-line therapeutic option in patients with early HCC^[7]. The restrictive approach indicated by Western guidelines was established more than 15 years ago^[21], and its conservative recommendations for surgical indications have not evolved over time despite the significant improvement in surgical techniques and technologies and their reflections on patient outcomes. An extension of the recommendations has been repeatedly suggested given that resection can be attempted with high rates of technical success and acceptable survival rates in patients with clinically significant portal hypertension^[22], multiple nodules^[10] or intrahepatic vascular invasion^[9]. In this context, it is not surprising to observe that experienced surgical centres both in

the East and in the West adopt a more liberal approach to LR in HCC that does not strictly follow the guidelines. In a recent large multicentre series of patients resected for HCC, less than 30% of cases were considered as ideal candidates for resection according to the current guidelines^[12].

In this study, we retrospectively analyzed a large series of approximately 1000 HCC patients who underwent LR at two hospitals in Milan (Italy) with large volumes of activity. Both centres offered LR even outside the current EASL/AASLD guidelines, and indeed greater than 60% of patients were considered as non-ideal candidates for LR. At baseline, patients presented with clinically relevant portal hypertension and/or abnormal bilirubin in greater than 30% of cases and had multifocal tumors in greater than 20% of cases. In addition, the maximum tumor size was larger than 5 cm in greater than 30% of cases. The low perioperative mortality of 3.5% observed at 3 mo and the long follow-up of nearly 60 mo allowed a thorough analysis of those preoperative factors that independently influenced the long-term survival of these patients. To reduce the bias deriving from the absence of an external validation cohort, the case series was randomly divided into a training set and a validation set. We then performed the uni- and multivariable analysis on the training set. As expected, the independent variables related to survival were liver related (MELD score > 9, presence of active HCV infection) and tumor related (number of nodules > 3, the largest diameter of nodules > 5 cm and presence of portal invasion). Interestingly, as previously observed^[22], clinically relevant portal hypertension did not independently affect survival. The same occurred for bilirubin above normal levels, which was not independently associated with survival when a composite score, such as MELD, was introduced in the multivariable analysis.

According to the weight of each factor independently related with survival and the corresponding risk estimates, an easy-to-determine prognostic scoring system was built. Then, according to changes in the risk estimates for each point increase, a stratification in three prognostic strata was computed: low (0-1 points), intermediate (2 points) and high (3-9 points) risk population. The corresponding median survivals were 77.2 mo (95%CI: 67.4-87.0), 41.7 mo (95%CI: 34.7-48.7) and 17.4 mo (95%CI: 10.1-24.6), respectively ($P < 0.0001$). The significant difference

in survival, overall and between strata, was confirmed also in the validation set and the entire cohort. This scoring system allows prospecting the post-surgical outcomes by assessing five easily accessible characteristics and thus may help clinicians when selecting between different treatment options for HCC patients.

Patients in the low-risk category were considered as ideal candidates for LR according to the observed survival of approximately 60% at 5 years, which approximates that of patients undergoing LT for HCC. This finding allowed to consider 593 patients (69.4% of the total of 854 evaluable patients) as “ideal candidates” for resection with respect to patients who would have been considered as “ideal candidates” according to EASL/AASLD guidelines (less than 40% patients) and resulted in a net increase of 31.6% of patients with indication for LR. The predictive power of the proposed criteria in the identification of the ideal candidate for resection was similar to that of the current guidelines in terms of AIC and Harrel’s C statistics. Most importantly, inclusion of a significantly increased number of patients in the definition of “ideal candidates” did not result in a significant decrease in terms of survival. After all, the proposed score broadens and enhances the concept of “surgical HCC” that is often discarded in the hepatology community due to an insufficient definition and poor evidence.

There are some limitations of this study. Firstly, despite prospective data collection, this is a retrospective study performed in only two high-volume centres. An external validation in a different population is required to strengthen the study results. Secondly, a different method of defining the training set could have been chosen, *e.g.*, the bootstrapping method, although the presented sample size was sufficiently large to meet generalizability criteria. Thirdly, active HCV infection was identified as an independent prognostic factor in this series, and this result may not totally apply in other settings where other aetiologies of cirrhosis are prevalent. In this respect, the recent introduction of direct antiviral agents to treat HCV infection may reveal other factors with a significant weight on patients’ prognosis in the future. Finally, this study included only patients who underwent open LR. Some factors, particularly those related to liver function, may have less significant impacts on long-term outcomes for patients undergoing laparoscopic LR^[23].

In conclusion, this study provides an easily accessible tool to stratify the prognosis of patients undergoing LR for HCC. The identified subset of patients at low risk could enter the group of ideal candidates for LR given that their prognosis approaches that of patients undergoing LT for HCC. The proposed criteria may expand safely the current EASL/AASLD indications for LR with no detrimental effect on patient prognosis.

COMMENTS

Background

The prognosis of patients with hepatocellular carcinoma (HCC) largely depends on tumor extension and underlying liver function. According to Western Guidelines, liver resection (LR) is considered as the first-line treatment only for a restricted subset of patients with an optimal liver function, a preserved physical condition and a single tumor nodule with no evidence of extra-hepatic spread or involvement of major vascular structures. If this profile is fulfilled, postoperative morbidity is low and long-term survival may equate that of liver transplantation.

Research frontiers

Several field practice studies have ascertained that LR is often offered outside Western guidelines, and various authors reported acceptable survival rates for patients with HCC resected at a more advanced stage because of macrovascular invasion, multiple nodules or impaired liver function. In addition, more recent studies demonstrate a survival benefit of radical surgery with respect to the available treatment alternatives across the different Barcelona Clinic Liver Cancer staging system stages.

Innovations and breakthroughs

The authors analyzed a large consecutive series of patients who underwent LR for HCC at two Italian centres, of whom greater than 60% of cases were outside Western guidelines. Five variables were identified as independently related to survival: Model for End-stage Liver Disease score > 9, presence of active hepatitis C virus infection, number of nodules > 3, largest diameter of nodules > 5 cm and presence of portal invasion. According to the weight of each variable, an easy-to-determine prognostic scoring system was built that allowed the identification of three risk strata with significantly different survival rates. Overall survival of patients in the low-risk strata was similar to that of patients who underwent LR according to Western guidelines. Considering LR patients in the low-risk strata as “ideal candidates” allowed a net increase of 31.6% of patients with indication for LR with respect to Western guidelines.

Applications

This scoring system allows assessment of the post-surgical outcomes by assessing five easily accessible characteristics and thus may help clinicians when selecting between different treatment options for HCC patients. Inclusion of a significantly higher number of patients in the definition of “ideal candidates” did not result in a significant decrease in terms of survival. Thus, the proposed score may broaden and enhance the concept of “surgical HCC” that is often discarded in the hepatology community due to an insufficient definition and poor evidence.

Terminology

Ideal candidates for LR according to Western guidelines are defined by an optimal liver function (Child-Pugh A, normal bilirubin and absence of clinically relevant portal hypertension), a preserved physical condition (ECOG Performance Status of 0), and a single tumor nodule with no evidence of extra-hepatic spread or involvement of major vascular structures.

Peer-review

The study is interesting and through a sophisticated statistical analysis of a large group of patients, provides a demonstration of the possibility to expand the obsolete European Association for the Study of the Liver/American Association for the Study of Liver Diseases guidelines. The topic is important and this is a well-organized study.

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Retrospective Study

Clinicopathological features of alpha-fetoprotein producing early gastric cancer with enteroblastic differentiation

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Abstract

AIM

To investigate clinicopathological features of early stage gastric cancer with enteroblastic differentiation (GCED).

METHODS

We retrospectively investigated data on 6 cases of early stage GCED and 186 cases of early stage conventional gastric cancer (CGC: well or moderately differentiated adenocarcinoma) who underwent endoscopic submucosal dissection or endoscopic mucosal resection from September 2011 to February 2015 in our hospital.

GCED was defined as a tumor having a primitive intestine-like structure composed of cuboidal or columnar cells with clear cytoplasm and immunohistochemical positivity for either alpha-fetoprotein, Glypican 3 or SALL4. The following were compared between GCED and CGC: age, gender, location and size of tumor, macroscopic type, ulceration, depth of invasion, lymphatic and venous invasion, positive horizontal and vertical margin, curative resection rate.

RESULTS

Six cases (5 males, 1 female; mean age 75.7 years; 6 lesions) of early gastric cancer with a GCED component and 186 cases (139 males, 47 females; mean age 72.7 years; 209 lesions) of early stage CGC were investigated. Mean tumor diameters were similar but rates of submucosal invasion, lymphatic invasion, venous invasion, and non-curative resection were higher in GCED than CGC (66.6% *vs* 11.4%, 33.3% *vs* 2.3%, 66.6% *vs* 0.4%, 83.3% *vs* 11% respectively, $P < 0.01$). Deep submucosal invasion was not revealed endoscopically or by preoperative biopsy. Histologically, in GCED the superficial mucosal layer was covered with a CGC component. The GCED component tended to exist in the deeper part of the mucosa to the submucosa by lymphatic and/or venous invasion, without severe stromal reaction. In addition, Glypican 3 was the most sensitive marker for GCED (positivity, 83.3%), immunohistochemically.

CONCLUSION

Even in the early stage GCED has high malignant potential, and preoperative diagnosis is considered difficult. Endoscopists and pathologists should know the clinicopathological features of this highly malignant type of cancer.

Key words: Alpha-fetoprotein-producing gastric cancer; Gastric cancer with enteroblastic differentiation; Early gastric cancer; Glypican 3; SALL4

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Core tip: We evaluated the comparison of clinicopathological features between 6 cases of early stage gastric cancer with enteroblastic differentiation (GCED) and 186 cases of early stage conventional gastric cancer (CGC: well/ moderately differentiated adenocarcinoma). Lymphatic, venous, and submucosal invasion rates were higher in GCED than CGC (33.3% *vs* 2.3%, 66.6% *vs* 0.4%, 66.6% *vs* 11.4% respectively, $P < 0.01$). In addition, Glypican 3 was the most sensitive marker for GCED (positivity, 83.3%), immunohistochemically. GCED has high malignant potential even at an early stage, and preoperative diagnosis is considered difficult. Further investigations are needed to establish optimal treatment approaches for GCED.

Matsumoto K, Ueyama H, Matsumoto K, Akazawa Y, Komori H, Takeda T, Murakami T, Asaoka D, Hojo M, Tomita N, Nagahara A, Kajiyama Y, Yao T, Watanabe S. Clinicopathological features of alpha-fetoprotein producing early gastric cancer with enteroblastic differentiation. *World J Gastroenterol* 2016; 22(36): 8203-8210 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i36/8203.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i36.8203>

INTRODUCTION

Gastric cancer with enteroblastic differentiation (GCED) was proposed as a very rare variant of alpha-fetoprotein-producing gastric cancer (AFPGC) and its clinicopathological features have not been well elucidated. There are few reports about GCED, and most are case reports. In these earlier reported cases, GCED was histologically characterized as having a primitive intestine-like structure composed of cuboidal or columnar cells with clear cytoplasm^[1-3]. Murakami *et al*^[4] reported 29 cases of GCED in which stages ranged from an early to an advanced stage based on clinicopathologic and immunohistochemical characteristics. They proposed that GCED showed aggressive behavior such as lymphatic and venous invasion, lymph node metastasis, and liver metastasis, and its clinicopathologic features were similar to those of AFPGC.

Recently, with the advent and widespread use of endoscopic submucosal dissection (ESD), indications for endoscopic treatment of early stage gastric cancer have been expanding rapidly. Therefore, it is necessary for endoscopists to know the clinicopathological features of various histological types of early gastric cancer. However, no report has focused on the early stage of GCED. This study aimed to clarify the clinicopathological features of early stage GCED by comparisons with the early stage of conventional gastric cancer (CGC), including well or moderately differentiated carcinoma.

MATERIALS AND METHODS

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Juntendo University School of Medicine Institutional Review Board.

All procedures were recorded in endoscopy databases on cases that underwent endoscopic resection in our hospital. The databases were examined to identify all cases of early stage GCED and CGC that underwent ESD or endoscopic mucosal resection from September 2011 to February 2015. We evaluated the comparison between early gastric cancer with a GCED component and early stage CGC clinicopathologically.

GCED was defined as a tumor having a primitive

Table 1 Clinicopathological findings for patients with gastric cancer with enteroblastic differentiation

Case	1	2	3	4	5	6
Age, yr	61	77	78	75	80	83
Male/female	Male	Male	Male	Male	Female	Male
Tumor location: U/M/L	L	M	U	U	L	M
Tumor size, mm	8	11	14	18	6	36
Macroscopic type	0-IIc	0-IIc	0-IIc	0-IIa	0-IIc	0-IIa + I
<i>H. pylori</i> infection	HPIgG+	HPIgG+	HPIgG-	NA	HPIgG+	HPIgG+
Procedure method	ESD	ESD	ESD with additional surgery	ESD	ESD	ESD with additional surgery
Depth of invasion (μm)	M	SM (1500)	SM (1000)	SM (200)	M	SM (2000)
Lymphatic invasion	(-)	(-)	(-)	(-)	(+)	(+)
Venous invasion	(-)	(+)	(+)	(+)	(-)	(+)
Observation period (mo)	51, ANED	42, ANED	N/A	38, ANED	N/A	28, ANED
Immunohistochemical analysis						
AFP	(+)	(-)	(-)	(-)	(-)	(-)
Glypican3	(+)	(+)	(+)	(+)	(-)	(+)
SALL4	(+)	(-)	(-)	(-)	(+)	(-)
MUC2	(-)	(-)	(-)	(+)	(-)	(-)
MUC5AC	(-)	(-)	(-)	(+)	(-)	(-)
MUC6	(-)	(+)	(+)	(+)	(-)	(+)
CD10	(+)	(+)	(+)	(+)	(+)	(+)
P53	(-)	(-)	(+)	(+)	(+)	(-)

U: Upper third of stomach; M: Middle third of stomach; L: Lower third of stomach; *H. pylori*: *Helicobacter pylori*; IgG: Immunoglobulin G; ESD: Endoscopic submucosal dissection; M: Mucosa; SM: Submucosa; ANED: Alive with no evidence of disease; N/A: Not applicable; AFP: Alpha-fetoprotein.

Table 2 Results of comparison between patients with gastric cancer with enteroblastic differentiation and conventional gastric cancer *n* (%)

	GCED	CGC	<i>P</i> value
Number of lesions, <i>n</i>	6 (2.7)	209 (97.3)	N/A
Age, yr; median (range)	75.7 (61-83)	72.7 (40-92)	0.39
Male/female	5/1	139/47	0.63
Tumor location: U/M/L	2/2/2	23/91/95	0.24
Tumor size, mm, mean (range)	15.0 (6-36)	15.2 (2-60)	0.96
Macroscopic type: elevated type/flat or depressed type	2/4	92/117	0.60
Ulceration	0	19 (9.0)	0.43
Depth of invasion: M/SM	2/4	185/24	< 0.01
Rate of submucosal invasive cancer	66.60%	11.40%	
Median SM invasive depth, μm (range)	1500(200-2000)	795.8(100-5000)	0.40
Lymphatic invasion	2 (33.3)	5 (2.3)	< 0.01
Venous invasion	4 (66.6)	1 (0.4)	< 0.01
Positive horizontal margin	0	9 (4.3)	0.60
Positive vertical margin	1 (16.7)	5 (2.3)	< 0.05
Curative resection ¹	1 (16.7)	186 (89.0)	< 0.01
The following is a comparison only for SM invasive cancer			
Number of SM invasive lesions, <i>n</i>	4	24	N/A
Lymphatic invasion	1 (25)	5 (20.8)	0.88
Venous invasion	4 (100)	1 (4.2)	< 0.01
Positive horizontal margin	0	1 (4.2)	0.33
Positive vertical margin	1 (16.7)	2 (8.3)	0.55
Curative resection ¹	0	12 (50)	< 0.01

¹Curative resection was according to Gastric Cancer Treatment Guidelines 2010 or 2014 provided by the Japanese Gastric Cancer Association. GCED: Gastric cancer with enteroblastic differentiation; CGC: Conventional gastric cancer (well or moderately differentiated carcinoma); U: Upper third of stomach; M: Middle third of stomach; L: Lower third of stomach; M: Mucosa; SM: Submucosa.

intestine-like structure composed of cuboidal or columnar cells with clear cytoplasm and immunohistochemical positivity for either alpha-fetoprotein (AFP) (rabbit polyclonal, 1:1000; Dako, Glostrup, Denmark), Glypican 3 (clone 1G12, 1:200; BioMosaics, Burlington, VT, United States) or SALL4 (clone 6E3, 1:100; Abnova, Taipei, Taiwan). Results of histology and immunohistochemical staining were evaluated by two pathologists specialized in the gastrointestinal tract. We determined curative resection criteria according to Gastric Cancer Treatment Guidelines 2010 or 2014 provided by the Japanese Gastric Cancer Association^[5,6].

Statistical analyses were conducted using SPSS (version 15.0 for Windows; SPSS Inc., Chicago, IL, United States) software. The comparison of clinicopathological features between GCEDs and CGCs were examined by the χ^2 test and the Mann-Whitney *U* test. The level of significance was set at *P* < 0.05.

RESULTS

This study included 192 cases (144 males, 48 females; mean age 72.8 ± 7 years; 215 lesions) of early stage GCED and CGC. Among 192 cases, there were 6 GCED cases (5 males, 1 female; mean age 75.7 years; 6 lesions) and 186 CGC cases (139 males, 47 females; mean age 72.7 years; 209 lesions). Table 1 shows the clinicopathological findings of the patients with GCED and Table 2 shows results of the comparison between patients with GCED and CGC. Four cases who were checked for *Helicobacter pylori* (*H. pylori*) infection by *H. pylori*-immunoglobulin G were positive. No significant differences were observed in tumor

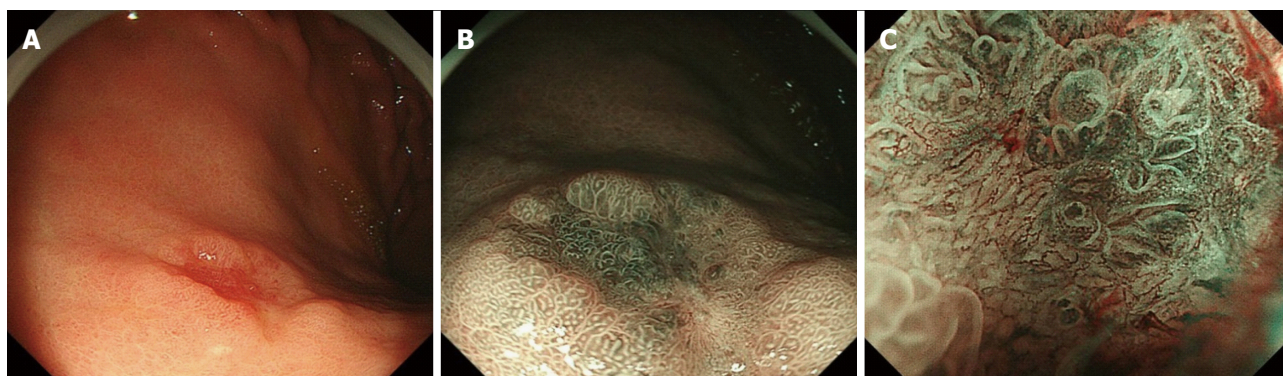


Figure 1 Endoscopic findings (case 1). A: Endoscopic examination with a white light image revealed a 10 mm reddish depressed lesion on the posterior wall in the middle third of the stomach. There were no specific features of deep submucosal invasion; B: Endoscopic examination with narrow band imaging (NBI). A demarcation line was clearly present between a depressed lesion and the surrounding mucosa; C: Magnifying endoscopy with NBI findings. Within the demarcation line, an irregular microvascular pattern (bizarre and tortuous vessel) and an irregular microsurface pattern (curved marginal crypt epithelium) are demonstrated. There were no specific features of GCED. GCED: Gastric cancer with enteroblastic differentiation.

location (U/M/L = 2/2/2 and 23/91/95 in GCED and CGC, respectively), mean tumor size (15.0 and 15.2 mm in GCED and CGC, respectively), and macroscopic types (flat or depressed type/ elevated type = 4/2 and 92/117 in GCED and CGC, respectively). Endoscopically, we could not find specific features of GCED or anticipate deep submucosal invasion (Figure 1). No lesion was diagnosed as GCED by examination of biopsy specimens. Regarding the depth of tumor invasion, the total submucosal invasion rate was significantly higher in GCED than CGC (66.6% vs 11.4%, $P < 0.01$). Positive rates for lymphatic and venous invasion were significantly higher in GCED than CGC (33.3% vs 2.3% and 66.6% vs 0.4%, $P < 0.01$). Therefore, the curative resection rate was significantly lower in GCED than CGC (16.7% vs 89.0%, $P < 0.01$). Moreover, when comparing only SM invasive cancer, the positive rate for venous invasion was significantly higher in GCED than CGC (100% vs 4.2%, $P < 0.01$) and the curative resection rate was significantly lower in GCED than CGC (0% vs 50%, $P < 0.01$). In 2 of the 6 GCED cases, additional surgery was performed (case 3: T1N0M0, stage IA; case 6: T1N1M0, stage IIA). Recurrence or metastasis was not seen in any of the 4 GCED cases that were followed from 28 to 51 mo (median 40 mo).

Histological examination of GCED demonstrated the presence of tubulopapillary carcinoma with clear cytoplasm at the deeper part of the mucosa and submucosa by lymphatic and/or venous invasion, and there was no severe stromal reaction. In all GCED cases, the superficial mucosal layer was covered with a conventional tubular adenocarcinoma (Figure 2). All GCED patients were positive for at least one of three enteroblastic lineage markers (AFP, Glypican 3, SALL 4). Glypican 3 was the most sensitive marker (positivity, 83.3%). Four cases were classified as having the gastrointestinal phenotype (4/6; 66.7%), and two as the intestinal type (2/6; 33.3%) according to combinations of the expression of CD10, MUC2,

MUC5AC, and MUC6 (Figure 3).

DISCUSSION

AFP was initially found in 1956 and is a fetal serum protein produced mainly in the fetal liver and yolk sac. After birth, AFP rapidly disappears and is not detected in the serum of healthy adults^[7]. The serum level of AFP is increased in some patients with hepatocellular carcinoma (HCC) or yolk sac tumor. In clinical practice, AFP has been considered to be a useful tumor marker in screening or monitoring patients with HCCs or yolk sac tumors. However, some diseases other than HCCs and yolk sac tumor were also associated with high serum levels of AFP, and gastric cancer is one of the most common^[2,8-15]. Since the first report of AFPGC by Bourreille *et al*^[16] in 1970, many cases have been reported, and the specifics of this disease have been gradually clarified.

Histologically, typical AFPGC has a liver-like structure and has been frequently reported as hepatoid adenocarcinoma^[17-20]. However, there are also histologic types of carcinoma other than hepatoid adenocarcinoma. In 1994, Matsunou *et al*^[1] reported two cases of AFP-producing gastric carcinoma with enteroblastic differentiation (AFP-producing clear cell gastric carcinoma), and the tumor characteristics were as follows: (1) columnar carcinoma cells growing primarily in tubulopapillary and glandular patterns; (2) abundant glycogen, but no mucin production in the clear cytoplasm; (3) gut hormone-containing cells scattered among clear carcinoma cells; (4) carcinoma cells producing oncofetal glycoproteins such as AFP and CEA; and (5) ultrastructurally, carcinoma cells showing well-developed microvilli with core filaments, whose rootlets formed occasional terminal webs, consistent with absorptive epithelium of fetal intestine or enteroblastic differentiation. Motoyama *et al*^[21] proposed that AFPGCs should be divided into three subtypes: (1) hepatoid type; (2) yolk sac type; and (3)

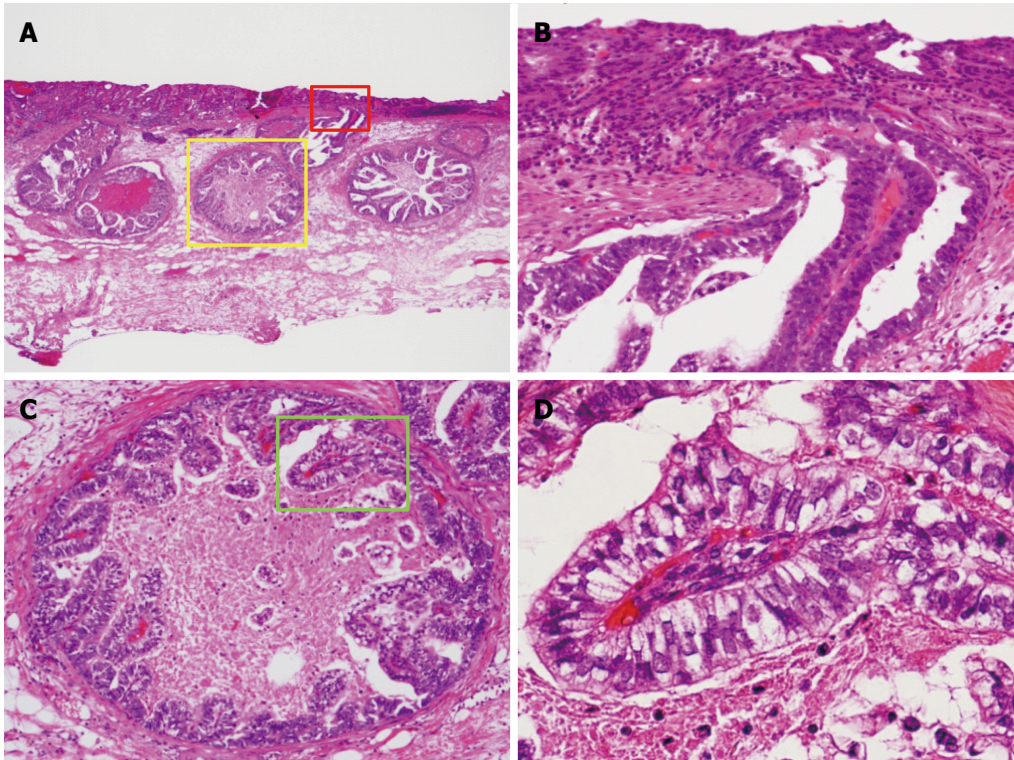


Figure 2 Histological examination of the resected specimens with hematoxylin and eosin stain (case 1). A and B: The superficial mucosal layer was covered with a well differentiated adenocarcinoma; B-D: Carcinoma cells with clear cytoplasm had tubulopapillary growth, resembling the primitive gut. These carcinoma cells arose from the deeper part of the mucosal layer and invaded into the submucosal layer by venous invasion. There was no severe stromal reaction at the submucosal layer. Pathological diagnosis was as follows: stomach (ESD): adenocarcinoma with enteroblastic differentiation, 0-IIc, 11 mm × 9 mm, well-differentiated carcinoma > moderately-differentiated carcinoma, papillary adenocarcinoma, SM (1500 μm), ly (-), v (+), HMO, VM0. SM: Submucosa; ESD: Endoscopic submucosal dissection.

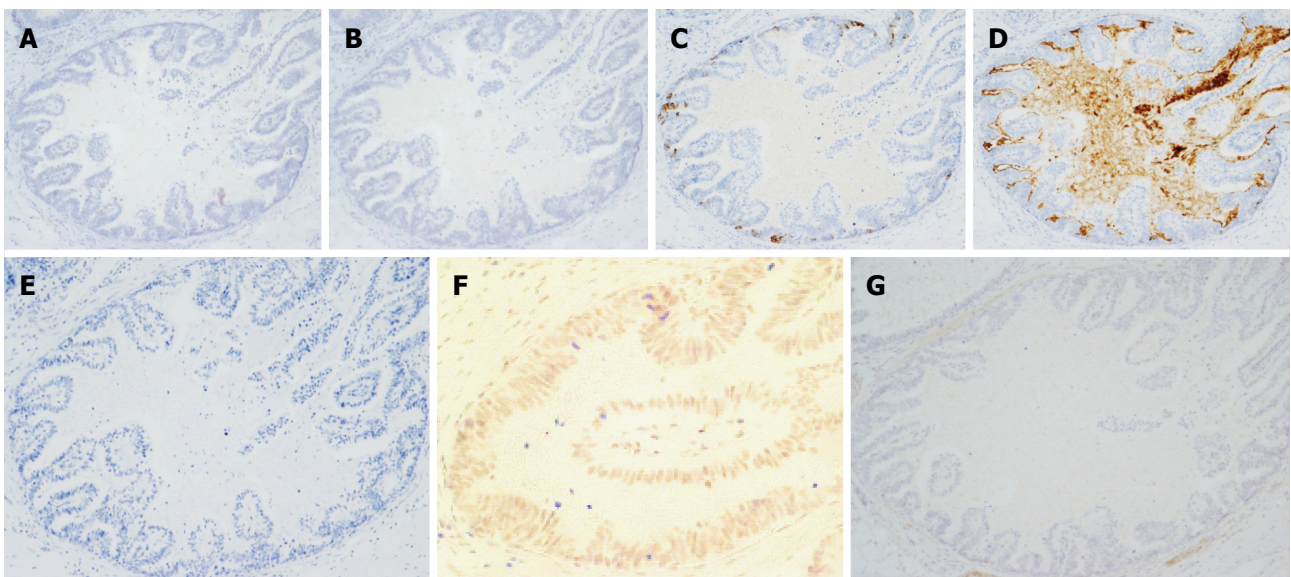


Figure 3 Immunohistochemical examination of resected specimens (case 1). A: MUC2; B: MUC5AC; C: MUC6; D: CD10; E: AFP; F: Glypican 3; G: SALL 4. The lesion had focal positivity for MUC6, diffuse positivity for CD10, weak positivity for Glypican 3 and negative staining for MUC2, MUC5AC, AFP and SALL4.

fetal gastrointestinal type. The fetal gastrointestinal type is thought to be equivalent to GCED^[1,3]. It is said that most AFPGCs have various mixtures of these histologic types and CGC. Kinjo *et al.*^[2] also reported that in a histologic type of submucosal invasive cancer of AFPGC, the positivity rates for CGC and GCED in

the mucosal component were significantly higher than in the invasive component. On the other hand, hepatoid gastric carcinoma (HGC) was significantly positive in the invasive component. In this study, pathological findings in all GCED cases showed that the mucosal layer was composed of conventional tubular

adenocarcinoma and GCED. In addition, the superficial mucosal layer was covered with a conventional tubular adenocarcinoma, and there were no findings of GCED components exposed to the superficial layer. Therefore, since we could only collect superficial mucosal layer tissue for the biopsy specimens, we felt that GCED could not be diagnosed by examination of biopsy specimens. The GCED component exists only in the deeper part of the mucosa to the submucosa by lymphatic and/or venous invasion but not in any other histological type of AFPGC (HGC and yolk sac type). As in previous reports^[2,4] these findings suggested that GCED could be thought to develop from CGC and then gradually develop hepatoid features during the process of tumor invasion and proliferation. We considered that there is a possibility that early stage GCED represents the early developmental stage of AFPGC, which has various mixtures of histological types.

Endoscopically, we diagnose the depth of invasion according to endoscopic features of gastric cancers with deep submucosal invasion (≥ 500 μm in depth), such as remarkable redness, uneven surface, margin elevation, ulceration, and enlarged folds^[22]. However, we could not anticipate the presence of deep submucosal invasion because none of these findings were revealed in our 6 GCED cases. The reason may be that there were few macroscopic changes in endoscopic morphology because the main submucosal invasive form of GCED depends on lymphatic and/or venous invasion and there were no severe stromal reactions.

In previous reports, AFPGC was usually associated with a poor prognosis with a high incidence of lymphatic invasion, venous invasion, and synchronous and metachronous liver metastasis^[23-25]. Hirasaki *et al.*^[26] studied 24 cases of AFP-producing early gastric cancer and reported that liver metastasis was seen in 2 cases (8.3%) and lymph node metastasis was seen in 18 of 24 cases (75.0%). From these results, they suggested that lymph node metastasis should be generally taken into consideration in AFP-producing early gastric cancer even if lymph node swelling is not seen on diagnostic imaging and that such patients should be under close periodic observation. Furthermore, Adachi *et al.*^[23] studied 270 cases of AFPGC and reported that there were 61 patients who underwent curative gastrectomy for stage I or II AFPGC, and that 39 of those patients (64%) were alive during a median follow-up period of 27 mo. From these results, it is difficult to say that the prognosis was good. Although in this study the mean diameters of GCED and CGC were about the same, comparisons of M and SM invasive cancer, positive rates of lymphatic and venous invasion, and non-curative resection rates were significantly higher in GCED than CGC. Furthermore, when comparing only SM invasive cancer, the positive rate of venous invasion was significantly higher in GCED than CGC (100% vs 4.2%, $P < 0.01$). Although lymph node swelling cannot

be seen on diagnostic imaging, lymph node metastasis occurred in 1 of 2 GCED cases who underwent additional surgery. In addition, although all of the 4 GCED cases for whom we could follow their complete progress were alive during the median follow-up period of 40 mo, 5 of 6 GCED cases (83.3%; 4 of 4 SM invasive cancer, 100%) had endoscopic non-curative resection because of deep submucosal invasion, lymphatic and/or venous invasion, and a positive margin. Therefore, we think that GCED, as well as AFPGC, may have a high incidence of liver and lymph node metastasis because of the high level of affinity to vessels and is associated with a poor prognosis in comparison with CGC. In this study, 2 of 5 cases who had endoscopic non-curative resection were followed up without additional surgery. One of the 5 cases had additional surgery and the remaining 2 were followed elsewhere. Although the 2 cases that we are following are alive without a recurrence (case 2: 42 mo, case 5: 38 mo), it is thought that close periodic observation is strictly required in such cases.

The present study had some limitations. Since GCED is a disease with a very low incidence rate, the number of patients was small. Furthermore, the data were collected retrospectively in a single medical center, which may produce selection bias.

In conclusion, GCED is rare, but has distinct clinicopathological features, especially in terms of high malignant potential and difficulty in preoperative diagnosis by biopsy specimens and prediction of submucosal invasion by endoscopic findings. It is necessary for endoscopists and pathologists to be aware of the clinicopathological features of such a rare and highly malignant type of cancer. In addition, when we detect specific histological findings for GCED with hematoxylin and eosin stain, immunohistochemical examination should be performed to make a precise diagnosis. However, there has been no report that evaluated a large number of cases. Further investigations are needed to elucidate the natural history of GCED by assessing the long-term outcome and establishing optimal treatment approaches for GCED.

COMMENTS

Background

Gastric cancer with enteroblastic differentiation (GCED) was proposed as a very rare variant of alpha-fetoprotein-producing gastric cancer (AFPGC). In previous reports, AFPGC was usually associated with a poor prognosis with a high incidence of lymphatic invasion, venous invasion, and synchronous and metachronous liver metastasis. Recently, with the advent and widespread use of endoscopic submucosal dissection (ESD), indications for endoscopic treatment of early stage gastric cancer have been expanding rapidly. Therefore, it is necessary for endoscopists to know the clinicopathological features of various histological types of early gastric cancer. However, the clinicopathological features and the optimal treatment approaches of GCED have not been well elucidated. In this study, we investigated clinicopathological features of early stage of GCED.

Research frontiers

There are few reports about GCED. Most of these are case reports, and there is no report that focused on the early stage of GCED. In these earlier reported cases, GCED was histologically characterized as having a primitive intestine-like structure composed of cuboidal or columnar cells with clear cytoplasm, and GCED showed aggressive behavior such as lymphatic and venous invasion, lymph node metastasis, and liver metastasis.

Innovations and breakthroughs

The results of this study contribute to clarifying the clinicopathological features of early stage GCED by comparisons with the early stage of conventional gastric cancer (CGC), including well or moderately differentiated carcinoma. In this study, even if it is early stage cancer, GCED has distinct clinicopathological features, especially in terms of high malignant potential with a high incidence of lymphatic and venous invasion, difficulty in preoperative diagnosis by biopsy specimens and prediction of submucosal invasion by endoscopic findings. In addition, Glypican 3 was the most sensitive marker for GCED (positivity, 83.3%), immunohistochemically.

Applications

If the authors detect specific histological findings for GCED with hematoxylin and eosin stain, immunohistochemical examination, especially Glypican 3 should be performed to make a precise diagnosis. However, there has been no report that evaluated a large number of cases. Further investigations are needed to elucidate the natural history of GCED by assessing the long-term outcome and establishing optimal treatment approaches for GCED.

Terminology

ESD is a well established technique of endoscopic resection that allows for en bloc removal of GI epithelial lesions. Glypican 3, it is an oncofetal protein that is expressed in the human fetal liver and placenta. It was discovered as a potential serological and histochemical marker whose expression is specific for hepatocellular carcinoma. SALL 4, it is an oncofetal protein that is expressed in the human fetal liver and silenced in the adult liver, but it is reexpressed in a subgroup of patients who have hepatocellular carcinoma and an unfavorable prognosis.

Peer-review

This most remarkable point of this manuscript is the analysis of the influence of this rare type of tumor and its bad prognosis in comparison to the conventional one that justifies the research in this subtype.

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Observational Study

FOCUS: Future of fecal calprotectin utility study in inflammatory bowel disease

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Informed consent statement: All study participants provided informed written consent prior to study enrollment.

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Abstract

AIM

To evaluate the perspective of gastroenterologists regarding the impact of fecal calprotectin (FC) on the management of patients with inflammatory bowel disease (IBD).

METHODS

Patients with known IBD or symptoms suggestive of IBD for whom the physician identified that FC would be clinically useful were recruited. Physicians completed

an online “pre survey” outlining their rationale for the test. After receipt of the test results, the physicians completed an online “post survey” to portray their perceived impact of the test result on patient management. Clinical outcomes for a subset of patients with follow-up data available beyond the completion of the “post survey” were collected and analyzed.

RESULTS

Of 373 test kits distributed, 290 were returned, resulting in 279 fully completed surveys. One hundred and ninety patients were known to have IBD; 147 (77%) with Crohn’s Disease, 43 (21%) Ulcerative Colitis and 5 (2%) IBD unclassified. Indications for FC testing included: 90 (32.2%) to differentiate a new diagnosis of IBD from Irritable Bowel Syndrome (IBS), 85 (30.5%) to distinguish symptoms of IBS from IBD in those known to have IBD and 104 (37.2%) as an objective measure of inflammation. FC levels resulted in a change in management 51.3% (143/279) of the time which included a significant reduction in the number of colonoscopies (118) performed ($P < 0.001$). Overall, 97.5% (272/279) of the time, the physicians found the test sufficiently useful that they would order it again in similar situations. Follow-up data was available for 172 patients with further support for the clinical utility of FC provided.

CONCLUSION

The FC test effected a change in management 51.3% of the time and receipt of the result was associated with a reduction in the number of colonoscopies performed.

Key words: Inflammatory bowel disease; Biomarkers; Fecal calprotectin; Colonoscopy; Physician perspective

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Core tip: Fecal calprotectin (FC) is a biomarker that provides a method of non-invasive assessment for intestinal inflammation. We evaluated the perspective of a group of gastroenterologists regarding the clinical use of FC, in particular the impact it has on the management of patients with inflammatory bowel disease (IBD). Patients with suspected or known IBD were recruited for study participation. A “pre survey” and “post survey” were completed by the physician prior to and after receipt of the FC result respectively. Of the 279 FC and surveys completed, FC levels resulted in a change in management 51.3% of the time and resulted in a significant reduction in colonoscopies performed.

Rosenfeld G, Greenup AJ, Round A, Takach O, Halparin L, Saadeddin A, Ho JK, Lee T, Enns R, Bressler B. FOCUS: Future of fecal calprotectin utility study in inflammatory bowel disease. *World J Gastroenterol* 2016; 22(36): 8211-8218 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i36/8211.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i36.8211>

INTRODUCTION

Calprotectin is a cytosolic protein of mucosal neutrophils of the colon and small bowel that are extruded into the gastrointestinal (GI) tract when neutrophils undergo apoptosis^[1]. Fecal calprotectin (FC) levels can be measured quantitatively in the stool from patients with mucosal inflammation in the bowel wall as seen in active inflammatory bowel disease (IBD)^[2]. In fact, FC levels have been shown to be closely correlated with endoscopic evidence of mucosal inflammation in the intestine^[3]. FC is a simple, non-invasive test that can be measured with an ELISA or a rapid detection device.

Calprotectin has previously been shown to be a reliable marker of mucosal inflammation and several studies have explored the use of FC to distinguish the symptoms of irritable bowel syndrome (IBS) from IBD^[4,5], to monitor response to therapy in patients with known IBD^[6] and to assess for mucosal healing^[7]. Furthermore, recent studies have evaluated the ability of FC to predict disease flares in patients with IBD in remission^[8-10] as well as in the surveillance for post-operative Crohn’s disease (CD) recurrence^[11].

While the use of FC in clinical practice is increasing, there are limited studies that have looked at the physician experience and perspective of the use FC for the differentiation of GI symptoms as IBS or IBD, as well as for the assessment of IBD activity. This study evaluated the impact of FC test results in the setting of the aforementioned indications on the medical management of patients and the need for colonoscopy in an outpatient clinical cohort, as well as patient outcomes stratified to FC test results.

MATERIALS AND METHODS

Patient population

This was a multicenter, prospective cohort study including adult community and academic gastroenterology practices in British Columbia (BC), Canada. Gastroenterologists who had an affiliation with the University of BC (UBC) were invited to participate. Clinicians identified patients for whom FC was considered to be an appropriate next step in diagnosis and/or management of symptoms. Patients with known ischemic colitis, infectious enterocolitis or colorectal cancer or who were pregnant were excluded. Other exclusions included a history of extensive bowel resection (subtotal colectomy or more than three bowel resections), an ostomy, an ileoanal pouch, concurrent daily use of non-steroidal anti-inflammatory drugs or the inability to collect a stool sample and return it within three days.

Eligible patients were invited to enroll by the attending gastroenterologist during clinical care from February 2012 to August 2013. Written informed consent was obtained and patients were provided with the Easy Sampler™ stool collection kit and written

instructions regarding collection of a first morning stool sample. Five to 15 g of stool were collected and processed within five days of collection.

The stool samples were prepared and analysed according to the manufacturer's instructions using the BÜHLMANN Quantum Blue™ Calprotectin kit (BÜHLMANN Laboratories AG, Schönenbuch, Switzerland). All samples were processed by a trained research assistant at the Gastrointestinal Research Institute in Vancouver, BC. FC levels were expressed as micrograms per gram of feces with a range of 100 to > 1800 µg/g. Physicians were informed that a FC level of > 250 µg/g was considered indicative of inflammatory activity, however it was at the discretion of the individual treating physician as to how to incorporate the FC result into patient management.

Data collection

After enrollment, but prior to receiving the FC result, the attending physician completed an online "pre-survey" using an internet based survey tool (Survey Monkey Inc., Palo Alto, CA, United States) (Supplementary Material 1). The baseline survey captured demographic data as well as any history of IBD, the indication for the test, concurrent investigations being ordered and current management of the symptoms.

During completion of the survey, the physician indicated the clinical scenario for FC testing, with the use of the following categories: (1) differentiation of GI symptoms between IBS and IBD in the absence of a known diagnosis of IBD; (2) differentiation of symptoms in patients known to have IBD; (3) assessment of response to therapy in a patient with IBD; and (4) objective marker of disease activity in a patient with IBD. Categories 3 and 4 were separated in the survey to clearly identify the indication for the test, however were subsequently combined as the indication of inflammatory disease activity assessment to aid with analysis. The FC result was then sent to the physician and the physician completed a "post-survey" using the same internet based survey tool (Supplementary Material 2). This survey was designed to assess the impact of the FC result on the diagnosis and management of the patient's symptoms. Outcomes according to survey responses are presented within Results under the sub-heading survey analysis.

The two surveys had not been used previously but were piloted by the primary investigator to ensure ease of use and that the surveys represented the actual management decisions of the physician. During the study, 20 patient charts were audited to ensure that the information in the survey accurately reflected the patient management.

Validity of the post-survey was further ensured by follow up of a subset of patients to determine if there was a deviation from the management proposed in the post-survey response. In particular, colonoscopy occurrence within six months following FC collection

was considered relevant with respect to reliability of colonoscopy deferral indicated by post-survey responses. For this subset of patients, medical records were retrospectively reviewed in November 2015, enabling follow-up extending to 26 mo after the final post-survey submission. Findings are presented in Results under the sub-heading of follow-up analysis.

To gain insight into the predictability of FC for future IBD outcomes, within the aforementioned patient subset, clinical events in the twelve months following obtainment of FC were also considered, in particular the need for steroids, immunomodulators, biologics, hospitalization or surgery.

The primary outcome was the frequency with which the FC result resulted in a change in the management of the patient as indicated by the pre- and post-surveys. Secondary outcomes included the deferral of colonoscopies as a result of the FC level, the proportion of events for which the physician found the FC level to be useful in the care of the patient and the association of the FC result to subsequent clinical, endoscopic and/or radiological outcomes.

The primary outcome of change in management was analysed according to a positive result being > 250 µg/g in addition to > 100 µg/g, given the heterogeneity in the indication for FC in the study cohort as well as the uncertainty in the current literature relating to what is considered a "positive" and "negative" FC. Within the follow up subset, a FC result of > 100 µg/g was considered positive.

Statistical analysis

As this pilot study was predominantly descriptive in nature and not designed to test a specific hypothesis, no sample size calculation was performed. Using the Fisher's exact and McNemar χ^2 tests respectively, there was comparison of the impact of a positive and negative FC test on a decision to change patient management and on the number of colonoscopies performed. Statistical calculations were performed using GraphPad Prism (GraphPad Software Inc., United States) and Statistical Analysis (Version 9.4, United States) software. The statistical methods were overseen by Dr. Terry Lee. (Centre for Health Evaluation and Outcome Sciences, St Paul's Hospital).

Ethics approval was obtained from the Providence Health Care and the UBC research ethics board and the trial was registered at ClinicalTrials.gov (NCT01676324). All of the authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

Baseline patient demographics

Seventeen gastroenterologists from eight different community and academic centers throughout BC distributed a total of 373 test kits. Seven of the 17 gastroenterologists were from academic centres. Two hundred and ninety (77.7%) kits were returned for

Table 1 Baseline patient demographics

Change management	Total	Yes	No	Unsure	Pos FC (> 250 µg/g)	Neg FC (< 250 µg/g)
<i>n</i>	279	143	121	15	81	198
Age	39.4 ± 13.5	38.9	40	40.5		
Disease location, <i>n</i> (%)						
Crohn's disease	147	56 (38.1)	88 (59.9)	3 (2.0)	55 (37.4)	92 (62.6)
UC	43	17 (39.5)	20 (46.5)	6 (14.0)	20 (46.5)	23 (53.5)
Current Rx						
5-ASA	44	16	24	4	18	26
Steroids	23	17	4	2	11	12
Biologics	58	32	24	2	24	34
IMM	63	35	24	4	28	35
None	136	67	61	8	24	112
Indication for FC test						
To diagnose IBD	90	40	45	5	7	83
To distinguish IBS from IBD	85	46	37	2	33	52
As an objective measure of disease activity	104	57	39	8	41	63

Patient demographics, indication for the test and the number of subjects with a positive or negative result and the impact on patient management. FC: Fecal calprotectin; UC: Ulcerative colitis; 5-ASA: 5-aminosalicylate; IMM: Immunomodulator; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome.

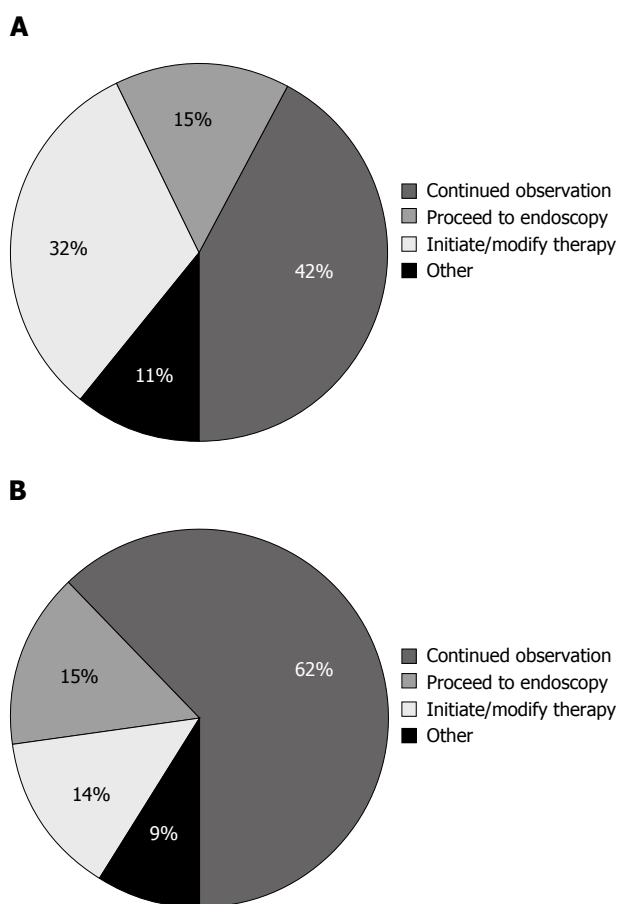


Figure 1 Management decisions following fecal calprotectin testing. Proportion of patients for each of the possible management options, A: When the fecal calprotectin (FC) test caused physicians to change the management of their patient (*n* = 143); B: When the FC test did NOT cause a change in patient management (*n* = 136).

processing resulting in 279 fully completed surveys. Of the 279 patients who returned the kits for processing, 177 (63.4%) were from academic centres.

The patients ranged between 19 and 79 years of age. One hundred and ninety (70%) were previously known to have IBD; 147 (77%) with CD, 43 (23%) with ulcerative colitis and 5 (3%) with IBD unclassified. The number of FC tests for each indication group were: 1.90 (32.2%) to differentiate a new diagnosis of IBS from IBD; 2.85 (30.5%) to distinguish GI symptoms due to active inflammation from IBS in those known to have IBD and 3.104 (37.2%) as an objective measure of disease activity in IBD. Of the 83 samples that were not returned, the indication for the test was to distinguish a new diagnosis of IBS from IBD in 31.3% (26/83), to assess abdominal symptoms in patients known to have IBD in 28.9% (24/83) and as a measure of inflammatory disease activity in 39.8% (33/83).

Survey analysis: Impact on patient management

Overall, FC test resulted in a change in management 51.3% of the time (143/279) according to the physician response to the post-survey. Table 1 shows the breakdown of the impact of FC on patient management based on the indication for performing the test, the disease type, the disease location and current treatment. Specific management decisions following receipt of FC test result are shown in Figure 1. When using either a cut-off of 250 or 100 µg/g, patients were significantly more likely to have a change in management when the FC result was positive (250 µg/g, $P < 0.0001$ (Figure 2)) (100 µg/g, $P = 0.0009$).

Survey analysis: Impact on colonoscopy

For 142 patients, the physician reported that if the FC test was not available, they would have performed a colonoscopy. After receipt of the FC result, a colonoscopy was planned according to the post-survey responses for 20 patients resulting in 122 colonoscopies (86%) that were not performed in favour of performing a FC test. Conversely, for 137 patients, a colonoscopy

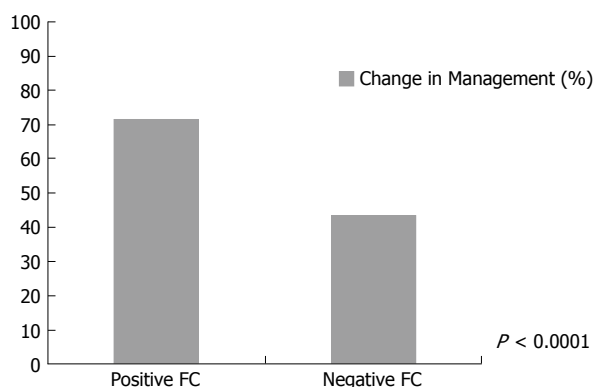


Figure 2 Impact of positive or negative fecal calprotectin result on management. Comparison of the percentage of patients undergoing a change in management as a function of whether the fecal calprotectin (FC) result was positive ($> 250 \mu\text{g/g}$) or negative ($< 250 \mu\text{g/g}$). Patients were significantly more likely to have a change in management when the FC result was positive ($P < 0.0001$).

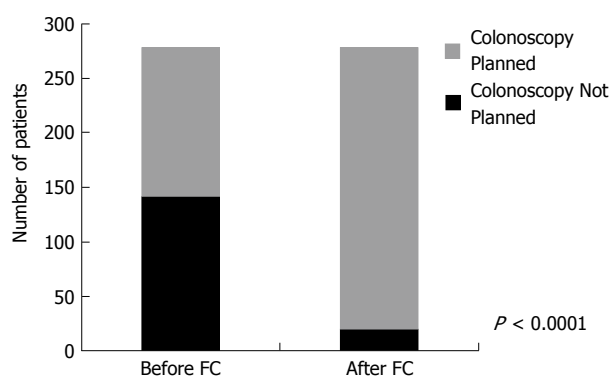


Figure 3 Impact of positive or negative fecal calprotectin result on colonoscopy occurrence. Reduction in colonoscopies planned as a result of the availability of the fecal calprotectin test. There was a significant reduction in colonoscopies planned ($P < 0.001$). FC: Fecal calprotectin.

was not planned, however, after receipt of the FC result, 4 patients underwent a colonoscopy as per the post-survey responses. Combining these two groups, there was a significant reduction in the total number of colonoscopies performed (118) due to the availability of the FC test ($P < 0.001$) (Figure 3).

Survey analysis: Impact on medical therapy

A change in medical therapy was planned for 36 patients, however, only six patients had a change in medical therapy after the physician received the FC result. Of the 243 patients for whom no change in therapy was planned, 41 underwent a change in their therapy after the FC test. For 60% (28/47) of the patients who underwent a change in therapy after the FC result, this change involved the initiation of a biologic (Infliximab or Adalimumab).

Survey analysis: Overall utility

Physicians reported the test to be sufficiently useful 97.5% of the time and to the extent that they would order it again for a similar patient in a similar clinical

situation. For the seven cases when the test was not felt to be useful, three FC results were greater than $250 \mu\text{g/g}$ (454, > 1800 , > 1800) and four less than $250 \mu\text{g/g}$ (< 100 , < 100 , 150, 233). Whether or not the FC level was positive (with either a cut off of > 100 or 250) did not significantly impact whether or not the physician found the test to be useful ($P = 0.4281$ for $> 250 \mu\text{g/g}$; $P = 0.1405$ for $> 100 \mu\text{g/g}$).

Follow-up analysis: Impact on colonoscopy and medical therapy

Medical record review was possible for 210 of the initial 373 patients recruited to the study. All of these patients had been recruited from an academic referral centre. For 172 of these 210 patients, FC testing and surveys were completed. (Figure 4) Of the remaining 38 patients who did not complete study FC testing, 31 patients were known to have IBD. Four of the 38 patients had endoscopic investigations instead at the discretion of the treating physician, while seven patients did not return for follow up after the initial recommendation for FC testing.

Review of available medical records for the 172 patients within the follow up subset indicated that for 33 patients there was deviation from the intended management that had been stated in the post-survey response. Accordingly, when considering the impact of FC on occurrence of colonoscopy, data pertaining to the number of colonoscopies that occurred in the six months following performance of FC within this follow-up subgroup was analyzed. As indicated by the pre-survey, the physicians reported that for 95 patients if the FC test was not available, they would have performed a colonoscopy. After receipt of the FC result, colonoscopy occurred in 24 of these patients over the subsequent six months and hence 72 colonoscopies (75%) were not performed as a result of the use of FC. Conversely, for 77 patients, a colonoscopy was not intended, however, after receipt of FC result, 11 patients underwent a colonoscopy. Combining these two groups, similarly to the original cohort analysis, there remained a significant reduction in the total number of colonoscopies performed ($P < 0.001$).

A degree of deviation between post-survey response and actual therapeutic management was detected during the follow-up analysis. Despite the post-survey responses indicating initiation or modification of medical therapy for 36 patients, this did not end up occurring for ten [therapy not initiated (8); not ceased (1); different immunomodulator started (1)].

Follow-up analysis: Impact on patient outcomes

Within the follow up cohort, 50 patients had undergone FC in the absence of an established diagnosis of IBD. Forty one patients had a FC result $< 100 \mu\text{g/g}$ of whom eight subsequently had normal endoscopic assessment. Of the remaining 33 patients with a FC $< 100 \mu\text{g/g}$, there have not been subsequent presentations and/or assessments to suggest an alternative diagnosis to IBS

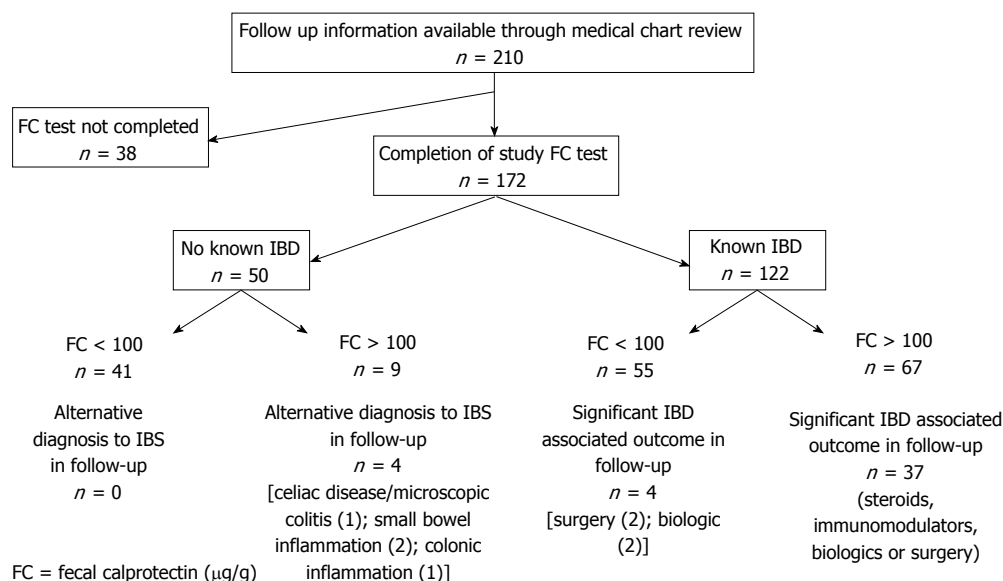


Figure 4 Outcomes of follow-up patient subset. Clinical outcomes in the follow-up subgroup according to FC result. FC: Fecal calprotectin; IBD: Inflammatory bowel disease.

since the FC testing. While for a proportion of patients there has been no further follow up at the academic centre since FC testing, this was considered likely to be reflective of no further clinical presentations given the nature of patients to be referred back to the same academic centre within the region.

Of the remaining nine patients with a FC result $> 100 \mu\text{g/g}$ in whom a diagnosis of IBS or IBD was yet to be established, four had abnormalities on subsequent investigations, while five have not had further investigations or presentations.

Within the IBD Cohort, 55 patients had a FC result $< 100 \mu\text{g/g}$. Of these patients, colonoscopy had been deferred in 24 following receipt the FC result. In 22 of these 24 patients there were no significant clinical outcomes recorded in the following 12 mo. One patient required surgery due to small bowel obstruction attributable to adhesions, and another patient had a CT enterography demonstrating active ileal disease for which an anti-TNF agent was commenced. Of the remaining 31 patients with a FC $< 100 \mu\text{g/g}$, two patients had discordant findings endoscopically. One patient was found to have active ileocolonic disease within six months, however there had been recent anti-TNF cessation and another patient had endoscopic detection of active ileal disease in the setting of post-operative recurrence surveillance, and was recommenced on anti-TNF therapy.

Of the 67 patients with a FC result $> 100 \mu\text{g/g}$, when reviewing the available medical records, there were clinically relevant outcomes over the following six months in 37 patients with either a need for steroids, immunomodulators, biologics, surgery or a combination of such management. Such changes had been captured in 23 of the post-survey responses.

DISCUSSION

The role of FC in GI disease assessment is continuing to evolve since it was first described in 1980, with such evolution including the indications for which FC is considered useful, as well as what defines the appropriate cut-off level. Accompanying such progression is ongoing debate as to the reliability and clinical significance of this biomarker.

Given persistent contention regarding the applicability of FC, this study aimed to systematically assess physician opinion of the utility of FC in the clinical management of patients with either suspected IBD or IBS or established IBD. Our results resonate the previously described utility of FC in this area of gastroenterology, with responding physicians indicating 97% of the time the test was sufficiently useful^[5,12]. Furthermore, the FC test impacted patient management more than half of the time with the greatest impact being an 86% reduction in the number of colonoscopies performed. Seventeen percent of patients had a change in their medical therapy as a result of the FC test and for 28 (60%) of these patients that change involved the initiation of a biologic. Such findings exemplify the high degree of utility of FC that was expressed by the study gastroenterologists.

There is mounting evidence that treatment of patients with IBD should be to a target of mucosal healing^[6,10,13] and not simply improvement in symptoms. This is due to a lack of correlation of symptoms with IBD activity, particularly for patients with CD. In the current study, 35 of 41 patients underwent a change in medical therapy for IBD after the physician received an elevated FC, implying that such patients were likely clinically quiescent and hence initially no change in

management was planned. However, receipt of the positive FC result resulted in a modification of therapy inferring that the goal of therapy was a reduction in mucosal inflammation and not just an improvement in symptoms, while also is likely reflective of the physician trust in FC as a marker of disease activity. As physicians gain more experience and confidence with FC results, one can anticipate even higher rates of change in medical therapy based on FC results.

Follow up of patient outcomes for a subgroup of the study population indicated that receipt of a FC result continued to minimize the need for colonoscopy for at least six months following completion of the post-survey, with 74% of the initially intended colonoscopies remaining deferred. Furthermore, follow up data also provided reassurance for the sensitivity of FC value of < 100 µg/g in the differentiation of IBS from IBD, with no suggestion of alternative diagnoses to IBS during follow up. However, a threshold of a FC result of 100 µg/g was at the expense of sensitivity, with five patients with a FC result greater than 100 µg/g continuing to have the most likely diagnosis of IBS during follow up. Previous studies have considered the diagnostic accuracy of FC levels to differentiate IBD from IBS, including Kennedy *et al.*^[5] who reported a sensitivity and specificity of 96% and 87% respectively when a FC threshold of 100 µg/g was used in an outpatient cohort of 895 patients. Within our follow up subgroup, a clinically significant change occurred in 55% of patients with IBD who had an elevated FC above 100 µg/g, which is consistent with several studies demonstrating the utility of an elevated FC for predicting clinical flare in IBD^[8-10]. Finally, the finding that a proportion of patients with a normal FC were subsequently found to have active small bowel disease is reflective of the known limitation of FC in this setting.

Targeting treatment to objective measures of disease activity in our current cost conscious environment is a very challenging task. An accurate and inexpensive marker of luminal inflammation such as FC may play a critical role in achieving targeted therapy. Overall, 118 colonoscopies were deferred as a result of the FC test in the current study. Previous studies looking at the cost of colonoscopy have estimated the costs to range between a low of \$352 in Canada to a high of \$2146 in the United States^[14,15]. The Canadian data is now approaching ten years old and taking the average cost reported in this study and adjusting for cost increases and inflation, one could conservatively estimate the cost to be at least \$500 in Canada. This represents nearly \$50000 saved on colonoscopy alone with the use of FC testing and does not consider any savings from optimization of medical management for patients with IBD (reduction of surgeries or hospitalization). Another study has also shown FC to be cost effective as a screening tool for IBD in both adults and children when the pre test probability was ≤ 75% and ≤ 65% respectively^[4].

Limitations to this study include potential bias

created by patient inclusion being solely dependent on the discretion of the involved physician. This accordingly has contributed to a heterogeneous patient population and is reflective of the limited guidance regarding the most appropriate use of FC at the time of study design. Given this was a real-life study, there was also unavoidable potential for inclusion of patients with co-existing undiagnosed conditions that could influence the FC level such as colonic polyps or additional immunologically driven digestive disorders. Another potential source of bias is that more than 20% of patients did not return FC samples. However, such patients were similarly distributed across the indications for the test suggesting that this did not influence the patient's decision to carry out the test. It is acknowledged that questionnaires were not previously validated although a degree of quality control was performed during the study to endeavor to ensure that the surveys accurately reflected the clinical practice of the doctors, with initial perusal of 20 surveys, and then a further follow up of 210 patients. The considerable duration of follow-up possible for the latter patients did enable adjustment for variation between what had been indicated by the physician on the post-survey response and what actually occurred.

Finally, at the time of initial study design, a FC level of 250 µg/g was considered appropriate for analysis of outcomes, however at the time of follow up analysis in 2015, a level of 100 µg/g was considered more appropriate. The "grey zone" between 100 and 250 µg/g and the accompanying need for additional investigations, particularly in IBD was highlighted in the recently published clinician guide to using FC to identify and monitor disease activity in IBD^[12].

In conclusions, FC is a simple, non-invasive test that is gaining widespread use in the diagnosis and management of IBD. This is the first Canadian data evaluating the role of FC in clinical practice, with demonstration that physicians find FC testing to be very useful not only for managing patients known to have IBD, but also to diagnose IBD in those with GI symptoms. This study also demonstrated the potential of FC to generate substantial cost savings to patients and the healthcare system in general. While the study was not designed to statistically assess clinical outcomes according to FC result, the descriptive findings are in concurrence with recommendations that FC can be used to differentiate between IBS and IBD, as well as be a guide to optimizing IBD therapy. It is hoped that studies such as this in addition to ongoing FC research pursuits will provide ongoing impetus for the availability and affordability of FC to clinicians and patients to be considered a priority by health service administrators.

COMMENTS

Background

The importance of objective, non-invasive assessment of luminal gastrointestinal (GI) symptoms in the diagnosis and management of inflammatory bowel

disease (IBD) is gaining increasing recognition. One strategy to meet such a need is the use of the biomarker, fecal calprotectin (FC).

Research frontiers

There is expanding literature regarding the use of FC, including differentiating symptoms of irritable bowel syndrome (IBS) from IBD, to monitor response to therapy in patients with known IBD and in the surveillance for post-operative Crohn's disease recurrence. Nonetheless, there is limited knowledge regarding the perspective of physicians using FC in the diagnostic and management algorithms for IBD with respect to potential impact on patient management.

Innovations and breakthrough

The authors present the findings of this study which considered the perceived utility of FC within an academic group of gastroenterologists. Physician perspective was obtained through the use of on-line surveys, while medical record review occurred in addition to this to determine clinical outcomes following FC testing. Faecal calprotectin was considered to have an impact on patient management in at least 50% of clinical circumstances in which it was used, including a reduction in the number of colonoscopies performed. Furthermore FC results and subsequent clinical outcomes provided support for the use of such a test in the differentiation of IBS from IBD as well as guiding therapeutic decisions in IBD.

Applications

This study supports the use of FC in clinical gastroenterology practice for the assessment of luminal GI symptoms to enable the differentiation of IBS and IBD and in the management of established IBD. This study also prompts further consideration of the potential cost-saving benefit of FC.

Terminology

FC is a cytosolic neutrophilic protein in the mucosa of the colon and small bowel which is released as a result of apoptosis. It can be measured quantitatively in the stool from patients with mucosal inflammation in the bowel wall as seen in active IBD.

Peer-review

The implementation of FC in clinical practice is a topic of importance and interest to the gastroenterology and wider primary care community. This manuscript is well written and the methodology, whilst imperfect, has characterised the role of calprotectin well in real world practice, showing a potential benefit in terms of cost reduction by reducing number of colonoscopies in this group.

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Observational Study

Pregnancy and inflammatory bowel disease: Do we provide enough patient education? A British study of 1324 women

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Informed consent statement: Patients provided informed consent by completing the anonymous online questionnaire.

Conflict-of-interest statement: Selinger CP has received unrestricted research grants from Warner Chilcott, and Abbvie, has provided consultancy to Warner Chilcott, Dr Falk, Abbvie and Takeda, and had speaker arrangements with Warner Chilcott, Dr Falk, Abbvie, MSD and Takeda. The other authors report no relevant conflict of interest.

Data sharing statement: Data are available on request from the lead author.

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Abstract

AIM

To examine patient knowledge and factors influencing knowledge about pregnancy in British women with inflammatory bowel disease (IBD).

METHODS

This is a post hoc analysis of a study of female members of Crohn's and Colitis United Kingdom, aged 18-45 years who were sent an online questionnaire recording patient demographics, education, employment, marital status, and disease characteristics. Disease related pregnancy knowledge was recorded using Crohn's and colitis pregnancy knowledge score (CCPKnow).

RESULTS

Of 1324 responders, 776 (59%) suffered from Crohn's disease, 496 (38%) from ulcerative colitis and 52 (4%) from IBD-uncategorised. CCPKnow scores were poor (0-7) in 50.8%, adequate (8-10) in 23.6%, good (11-13) in 17.7% and very good (≥ 14) in 7.8%. Multiple linear regression analysis revealed that higher CCPKnow scores were independently associated with higher

educational achievement ($P < 0.001$), younger age at diagnosis ($P = 0.003$) and having consulted a health care professional about pregnancy and IBD ($P = 0.001$).

CONCLUSION

Knowledge was poor in 50%. Speaking with health-care professionals was a modifiable factor associated with better knowledge. This illustrates the importance of disease related pregnancy education

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Patient knowledge; Pregnancy; Reproduction

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Core tip: Inflammatory bowel disease (IBD) affects many women of childbearing age and knowledge of IBD and pregnancy related issues, is key to enable patients to make informed decisions. In this large study of British women with IBD, knowledge was poor in over half of participants. Speaking with health care professionals about pregnancy was identified as a modifiable factor associated with better knowledge. This study illustrates first the importance of disease related education for female patients with IBD and second highlights that health professionals should seek opportunities to educate patients about pregnancy and IBD early in their disease course.

Carbery I, Ghorayeb J, Madill A, Selinger CP. Pregnancy and inflammatory bowel disease: Do we provide enough patient education? A British study of 1324 women. *World J Gastroenterol* 2016; 22(36): 8219-8225 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i36/8219.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i36.8219>

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disease and includes ulcerative colitis (UC), Crohn's disease (CD) and IBD-unclassified (IBD-U). Severity of disease can differ and the impact upon the quality of life for individual patients can vary dramatically, with one study reporting that up to 48% of patients feel IBD symptoms significantly affect their lives even whilst in remission^[1]. As the age of onset for IBD is usually around the reproductive years, questions about having children and being pregnant are common for many women who have IBD^[2]. Decision-making regarding IBD and pregnancy is complex and requires weighing the benefits and risks of treatments.

Fertility in female patients with IBD is generally equal or only marginally reduced when compared to the general population^[3-6]. Nevertheless, 42.7% of women with IBD fear infertility^[7] and studies have shown that voluntary childlessness is more common in

women with IBD than the general population^[8].

Recently, the role of IBD and pregnancy related patient knowledge has been scrutinized. The Crohn's and colitis pregnancy knowledge score (CCPKnow) is a validated self-report tool that has been developed for use in clinical practice as well as research^[9]. Its use has shown that whilst women with children are more likely to have higher CCPKnow scores^[9,10], those who are voluntarily childless tend to have lower scores^[11]. Importantly, poor knowledge has also been correlated with views about IBD medication that contradict medical guidelines^[12]. Making uninformed choices on IBD medication could lead to disease flares and potentially adverse pregnancy outcomes. Good disease pregnancy related knowledge is therefore key to women having a safe and successful pregnancy.

Average CCPKnow scores have been demonstrated to be between 5.4-7.47, with between 44.8%-65% scoring "poor" (CCPKnow < 8) in British and Australian cohorts^[9,11,13]. Previous studies have revealed some of the factors associated with higher CCPKnow scores including Caucasian ethnicity, higher income, having a partner, being a member of Crohn's and Colitis Patient Association and longer disease duration^[9]. However, these studies sampled a relatively small cohort of patients (< 250) and showed limited evidence into what modifiable factors might affect knowledge levels. The extent of IBD and pregnancy related patient knowledge in British women has so far not been investigated.

In light of the established correlation being between IBD patients' level of knowledge on pregnancy matters and their subsequent decisions on family planning one can highlight the importance of identifying factors affecting women's knowledge. The aim of this study is to ascertain the extent of low levels of knowledge amongst a sample of British women and to determine which factors, particularly which modifiable factors, influence knowledge of IBD and pregnancy.

MATERIALS AND METHODS

This study is a post hoc analysis of a recently published paper examining factors affecting voluntary childlessness in IBD^[11]. We contacted all 4300 female members of Crohn's and Colitis United Kingdom (the national charity providing support and patient education in addition to funding research for patients affected by IBD in the United Kingdom) aged 18-45 years by email. These women were then invited to complete our online questionnaire using an online survey system (Bristol Online Surveys, United Kingdom). Each received two invitation reminders and submission of the completed questionnaire was taken as informed consent.

The study collected self reported data on patients' demographics, educational achievements, employment status, marital status and sexual orientation. Furthermore, questions related to disease characteristics

Table 1 Patient demographics *n* (%)

Patient demographics	Value
Age (yr)	Mean 33.5 (18-45)
Ethnicity	
White	1258 (95.0)
Asian	34 (2.6)
Black	6 (0.5)
Other	26 (1.9)
Highest educational achievement	
Secondary school	157 (11.9)
Apprenticeship/guild/NVQ	386 (29.1)
Bachelor	577 (43.6)
Master/PhD	204 (15.4)
Employment status	
Full time employment	697 (52.6)
Part time employment	337 (25.5)
Full time education	102 (7.7)
Unemployed	80 (6.0)
House person	108 (8.2)
Relationship status	
Single	259 (19.6)
Separated/divorced	44 (3.3)
Long term relationship/married/civil partnership	1009 (76.2)
Other	12 (0.9)
Same sex relationship	
Yes	66 (5.0)
No	1250 (94.4)
Chose not to answer	8 (0.6)

consisted of diagnosis, duration of illness, and number of hospitalisations, surgical resections, and current as well as previous exposure to IBD medication. For the purpose of this post-hoc analysis each participant's child status was analysed as a dichotomous variable. As such, child status group 1 consisted of women who either had children after diagnosis of IBD or those who were planning to have children at some stage after diagnosis. Child status group 2 consisted of women who only had children prior to diagnosis, those who were experiencing infertility and those who decided to remain voluntarily childless.

The validated CCPKnow was used to assess disease-related pregnancy knowledge^[9]. This 17-item self-report questionnaire classifies knowledge as poor (0-7), adequate (8-10), good (11-13), and very good (14-17).

Data analysis was performed using SPSS 22.0 (IBM, Armonk, United States). We conducted ANOVAs and student *t*-tests for continuous data and chi-square tests for categorical data at a *P*-value < 0.05. To determine independent predictors of CCPKnow independence of variables was determined by entering any significant variables from the univariate analysis into multiple linear regression analysis. The study was approved by the National Research Ethics Service Committee North West - Preston (14/NW/1391).

RESULTS

A total of 1324 women completed the survey (response rate 31%) with a mean age of 33 years. Of these 1009 (76.2%) were in a long-term relationship, 259 (19.6%)

Table 2 Disease and treatment characteristics *n* (%)

Characteristics	Value
Diagnosis	
CD	776 (58.6)
UC	496 (37.5)
IBD-U	48 (3.6)
Unknown	4 (0.3)
Age at diagnosis	Mean 25 yr
	Median 24 yr
Duration of disease	Mean 8.5 yr
	Median 7 yr
Hospital admissions	
Yes	923 (69.3)
No	401 (30.3)
	Median 2 admissions
Bowel resection surgery	
Yes	437 (32.3)
No	897 (67.7)
	Median 2 resections
5-ASA medication	
Current	615 (46.4)
Previous	521 (39.4)
Never	188 (14.2)
Corticosteroids	
Current	175 (13.2)
Previous	948 (71.6)
Never	201 (15.2)
Immunomodulators	
Current	597 (45.1)
Previous	368 (27.7)
Never	359 (27.2)
Anti-TNF agents	
Current	280 (21.1)
Previous	196 (14.8)
Never	848 (64.1)

CD: Crohn's disease; IBD-U: Inflammatory bowel disease-unclassified; UC: Ulcerative colitis; TNF: Tumor necrosis factor.

were single and 44 (3.3%) were separated/divorced. Over half of patients had university level education with 577 (43.6%) having a bachelors degree and 204 (15.4%) having a masters or doctorate. Over three quarters were in employment, with 697 (52.6%) in full time employment, 337 (25.5%) in part time employment and only 80 (6%) unemployed. For further details on demographic variables please see Table 1.

Of the 1324 responders 776 (59%) suffered from CD, 496 (38%) from UC and 52 (4%) from IBD-U. Ninehundredtwentythree (69.3%) had been admitted to hospital due to their disease and 437 (32.3%) had undergone bowel resection surgery. Fourhundredseventysix (35.9%) were either currently taking or had previously been on biologic medications, 965 (72.8%) for immunomodulators and 1136 (85.8%) for 5-aminosalicylic acid medication with current or previous steroid use 1123 (84.8%). Detailed information regarding disease characteristics and medication history is available in Table 2.

CCPKnow scores were poor (0-7) in 673 participants (50.8%), adequate (8-10) in 313 participants (23.6%), good (11-13) in 235 participants (17.7%)

Table 3 Patient demographics, disease characteristics and impact of speaking to healthcare professionals on Crohn's and colitis pregnancy knowledge score

Factor	Groups	CCPKnow score	Significance
Age	N/A	Pearson correlation -0.131	$P < 0.001$
Age at diagnosis	N/A	Pearson correlation -0.212	$P < 0.001$
Ethnicity	White	7.32	$P = 0.773$
	Mixed	8.00	
	Asian	6.59	
	Black	6.33	
	Other	7.43	
Education	Secondary school	5.99	$P < 0.001$
	NVQ/diploma	6.08	
	Bachelors degree	8.02	
	Masters/PhD	8.67	
Relationship status	Single	6.26	$P < 0.001$
	Separated/divorced	6.82	
	Long term relationship	7.60	
	Not stated	7.67	
Employment status	Full-time	7.18	$P = 0.30$
	Part-time	7.73	
	Full-time education	7.46	
	Unemployed	6.12	
	House person	7.59	
Same-sex relationship	Yes	6.71	$P = 0.451$
	No	7.35	
	Chose not to answer	6.63	
Diagnosis	CD	7.51	$P = 0.051$
	UC	6.97	
	IBD-U	7.85	
	Unknown	4.25	
Children	Group 1	7.71	$P = 0.007$
	Group 2	7.05	
Partner with IBD	Yes	8.64	$P = 0.241$
	No	7.30	
Hospital admissions	Admission	7.59	$P < 0.001$
	None	6.66	
Resection surgery	Yes	7.71	$P = 0.018$
	No	7.12	
Current 5-ASA prescription	Yes	7.06	$P = 0.048$
	No	7.53	
Current immunomodulatory prescription	Yes	7.67	$P = 0.006$
	No	7.02	
Current anti-TNF prescription	Yes	8.30	$P < 0.0001$
	No	7.05	
Spoken to HCP	Yes	8.75	$P < 0.001$
	No	5.82	
Spoken to GP	Yes	8.44	$P < 0.001$
	No	6.98	
Spoken to gastroenterologist	Yes	9.18	$P < 0.001$
	No	6.25	
Spoken to IBD nurse	Yes	9.27	$P < 0.001$
	No	6.83	

CD: Crohn's disease; IBD-U: Inflammatory bowel disease-unclassified; UC: Ulcerative colitis.

and very good (≥ 14) in 103 participants (7.8%). Higher CCPKnow scores were associated with higher educational achievement, particularly postgraduate achievements vs high school achievements (Masters/PhD 8.67 vs Secondary school degree 5.99, $P < 0.001$). Working full time and being in a long-term relationship was respectively associated with better scores compared to being unemployed and being single (full time employment 7.18 vs unemployed 6.12, $P = 0.03$; long-term relationship 7.60 vs single 6.26, $P < 0.001$). Having children after being diagnosed with IBD or planning to have children was associated (group 1, CCPKnow = 7.71) with significantly better CCPKnow scores than having children prior to diagnosis, being infertile or planning to remain childless (group 2, CCPKnow = 7.05, $P = 0.007$). Patients with CD compared to UC patients, as well as (7.51 vs UC 6.97, $P = 0.026$) patients with markers of more severe disease (hospital admission 7.59 vs none 6.66, $P < 0.001$; surgery 7.71 vs none 7.12, $P = 0.018$; current biological therapy 8.30 vs none 7.05, $P < 0.001$) had higher knowledge scores. Ethnicity and same sex relationships had no influence on CCPKnow scores. We found significant but modest negative correlations between age and CCPKnow (Pearson correlation -0.131, $P < 0.001$) and between age at diagnosis and CCPKnow (Pearson correlation -0.212, $P < 0.001$). Participants of younger age and participants with younger age at diagnosis had better CCPKnow scores. See Table 3 for further details regarding CCPKnow scores.

Speaking to health care professionals about IBD and pregnancy, was also associated with better CCPKnow scores (spoken to a healthcare professional 8.75 vs not 5.82, $P < 0.001$). This significant difference persisted even when data was divided into specific types of healthcare professionals. Thus, talking to any healthcare professional significantly correlated with higher knowledge (Gastroenterologist 9.18 vs no 6.25, $P < 0.001$), (GP 8.44 vs no 6.98, $P < 0.001$), (Specialist IBD nurse 9.27 vs 6.83, $P < 0.001$; see Figure 1).

Multiple linear regression analysis revealed that university level education ($\beta = 0.292$, $P < 0.0001$), having spoken to a health care professional ($\beta = 0.317$, $P < 0.0001$) and younger age at diagnosis (increasing age $\beta = -0.274$, $P = 0.003$) were independently associated with higher CCPKnow scores (Table 4).

DISCUSSION

The importance of IBD and pregnancy related patient knowledge (as measured by CCPKnow) has been demonstrated in a number of key studies over the last 5 years. As discussed, poorer patient knowledge has been associated with inaccurate opinions about medications, which if acted upon could increase the risk of adverse IBD and potentially pregnancy outcomes^[12].

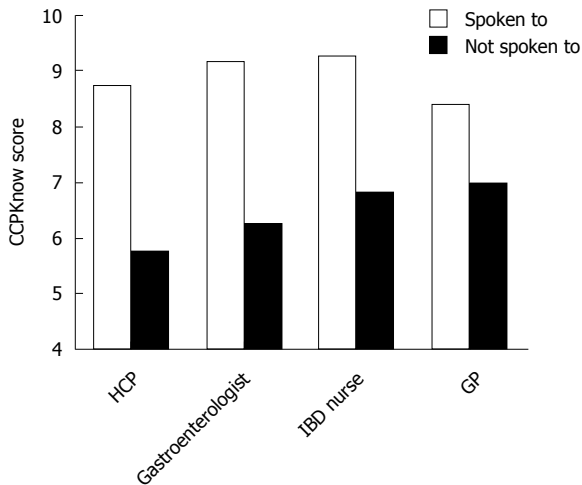


Figure 1 Demonstrates effect of talking to healthcare professionals on Crohn's and colitis pregnancy knowledge scores.

Importantly, poor IBD and pregnancy related patient knowledge is also associated with voluntary childlessness^[10,11]. Studies examining CCPKnow have so far reported on medium size cohorts only and data from the United Kingdom have previously been lacking. This is the largest study of CCPKnow so far and the first to study a cohort of British women with IBD. We have demonstrated that knowledge was poor in over half of patients.

While the number of studies examining IBD related pregnancy knowledge is still limited, the relationship between general patient knowledge in IBD patients and their physical and mental health outcomes has been investigated in a number of studies. Poor general IBD knowledge has been linked to patients having less adaptive coping strategies^[14]. It has also been hypothesised that better knowledge of general IBD issues might be associated with fewer disease complications, yet, Eaden *et al.*^[15] found no significant difference in knowledge between those who developed colorectal cancer as a complication of IBD and those patients who did not. However, as they discuss, their results may be skewed by the theory that having colorectal cancer itself may increase knowledge late in the disease course and perhaps comparing CCPKnow scores between patients who take up screening colonoscopies and those who do not would have demonstrated a difference.

Interestingly, better IBD knowledge has not always been associated with positive patient outcomes. Selinger *et al.*^[16] showed that better knowledge was associated with higher anxiety levels. Whilst it was not clear whether this was because more anxious patients sought out more knowledge, it is worth bearing in mind that by educating patients, we might potentially cause anxiety.

It is important to examine potential factors associated with poor IBD related patient knowledge. Such factors may help identify those patients most

Table 4 Multiple linear regression analysis

Factors	Beta-coefficient	Significance
Current 5-ASA (yes/no)	-0.490	$P = 0.603$
Current immunomodulators (yes/no)	-0.380	$P = 0.663$
Current biologics (yes/no)	0.111	$P = 0.201$
Educational achievement (university/no university)	0.292	$P = 0.001$
Work status (employed/unemployed)	-0.075	$P = 0.369$
Marital status (relationship/single)	0.120	$P = 0.147$
Child status (group 1/group 2)	-0.142	$P = 0.157$
Diagnosis (CD/UC)	0.090	$P = 0.336$
Surgery (yes/no)	-0.084	$P = 0.426$
Hospital admission (yes/no)	0.038	$P = 0.676$
Spoken to HCP (yes/no)	-0.317	$P < 0.0001$
Age	-0.211	$P = 0.053$
Age at diagnosis	-0.274	$P = 0.003$

in need of disease education, increase our understanding how patient knowledge is formed and in turn establish which interventions show potential to improve knowledge for women with IBD. Our study demonstrates that non-modifiable factors including education, employment and being in a relationship had a positive influence on CCPKnow score. It is not surprising that higher educational achievement was an independent predictor of higher CCPKnow scores and that pregnancy related knowledge is more relevant for women in a relationship compared to single women. This has been supported by previous studies^[9]. Patients with CD and higher disease burden also had better knowledge. While the exact reason for this cannot be determined from our data, the increased contact with healthcare professionals associated with more severe disease may have led to increased knowledge. Patients with more severe disease may also have a vested interest in knowing more, as it is likely that it will take more personal and professional effort to control their symptoms than someone with mild disease.

Not surprisingly having children after being diagnosed with IBD or planning to have children was associated with better CCPKnow scores. Some of the effects are likely related to the information provided during pregnancy care or sought out by the patients in the process of considering pregnancy. The effects of disease type, severity and child status on CCPKnow scores disappear on regression analysis suggesting that the most important factors influencing CCPKnow relate to general education achievement, age at diagnosis and having spoken to health care professionals. Child status was not an independent predictor of CCPKnow scores as there were clear connections between child status and age at diagnosis (older women were less likely to have children post diagnosis).

Speaking with health care professionals about pregnancy was identified as a strong and independent modifiable factor associated with better knowledge. Optimistically, perhaps this is largely due to the education patients receive during each consultation, however it

is worth considering that more knowledgeable patients may also demonstrate better attendance or seek more contacts with professionals. Indeed a single education seminar can improve CCPKnow score significantly^[13]. It is likely that pregnancy related IBD education can have positive effects on child planning, maternal and foetal outcomes as recently demonstrated in a Dutch study^[17]. Patients exposed to pre-conceptual counselling had better outcomes than those without such counselling^[17].

The positive contribution of patient education on clinical outcomes has been clearly demonstrated in other diseases. A study of an educational intervention for patients with diabetes showed not only increased knowledge scores, but also improved HbA1c levels and improved self-care and dietary practice^[18]. Similar results have been demonstrated following a patient education intervention for asthmatics; as knowledge increased, antibiotic use, steroid use and unplanned visits to the doctor all reduced^[19]. However, whilst patient education programmes almost universally show better knowledge levels, a study by Waters *et al.*^[20] was grossly underpowered and hence failed to show that an education programme for IBD patients influences medication adherence or quality of life.

Our study has a number of strength and weaknesses. This large sample of British women with IBD has a similar distribution of disease compared to other UK IBD cohorts; however more women were in long-term relationships and had university level education than in the United Kingdom adult population^[21,22]. As we have shown, higher educational level equates with higher CCPKnow scores, it is likely that the study samples' CCPKnow scores are actually a slight overestimation rather than underestimation compared to the general IBD population.

There are several limitations to this study. Firstly, all patients are members of Crohn's and Colitis United Kingdom, and membership has been shown to increase knowledge and specifically CCPKnow scores^[9]. We may have, therefore, overestimated CCPKnow scores somewhat. However, whilst this makes it likely that all studied patients had higher knowledge levels than found in non-members, our results showing differences in knowledge level still remain valid as we compared patients like for like and analysis focussed on differences influenced by other patient and disease characteristics.

Additionally, data collected regarding disease characteristics was self-reported and the anonymous study design did not allow for independent verification of such self reported data.

In conclusion we have demonstrated poor knowledge pregnancy related IBD patient knowledge in over half of this cohort. The positive impact speaking to a healthcare professional has on CCPKnow score is highly significant. This study therefore illustrates the importance of disease related education for female patients with IBD. Health professionals, of

any vocation, should seek opportunities to educate women of reproductive age about pregnancy and IBD early in their disease course in an effort to try reduce voluntary childlessness due to limited knowledge and/or incorrect assumptions and improve maternal and foetal outcomes.

COMMENTS

Background

Inflammatory bowel disease (IBD) affects many women of childbearing age and knowledge of IBD and pregnancy related issues, is key to enable patients to make informed decisions. This study examined knowledge and factors influencing knowledge about pregnancy in British women with IBD.

Research frontiers

The effects of patient knowledge on decision making regarding pregnancy, adherence to medical treatment during pregnancy and on foetal and maternal outcomes are all being studied currently. It is likely that patient knowledge plays a vital role in all of them.

Innovations and breakthroughs

This study demonstrates for the first time in a very large study that knowledge is poor in 50% of patients. The study has shown that speaking with health care professionals is associated with improved knowledge.

Applications

Women with IBD should be educated about pregnancy related issues early in their disease course to avoid voluntary childlessness. Pre-conceptual counselling should be available for women with IBD during their reproductive years.

Terminology

IBD are chronic inflammatory bowel diseases comprising Crohn's disease and ulcerative colitis.

Peer-review

The present manuscript reports the results of online survey regarding disease-related pregnancy knowledge in a relatively large sample of women with IBD from across the United Kingdom. They concluded disease-related education is important and health care professional should try to educate patients earlier. The manuscript is interesting and well written. Study methods and conclusion is clear. It will be helpful for readers.

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Clinical guidelines of non-alcoholic fatty liver disease: A systematic review

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Abstract

AIM

To perform a systematic review to grade guidelines and present recommendations for clinical management of non-alcoholic fatty liver disease (NAFLD).

METHODS

A database search was conducted on PubMed for guidelines published before May 2016, supplemented by reviewing relevant websites. The Appraisal of Guidelines for Research and Evaluation (ARGEE) Instrument II was a tool designed to appraise the methodological rigor and transparency in which a clinical guideline is developed and it is used internationally. It was used to appraise the quality of guidelines in this study. The inclusion criteria include: clinical NAFLD guidelines for adults, published in English, and released by governmental agencies or key organizations.

RESULTS

Eleven guidelines were included in this study. Since 2007, guidelines have been released in Asia (3 in China, 1 in South Korea, and 1 in Japan), Europe (1 in Italy),

America (1 in United States and 1 in Chile) and three international agencies [European associations joint, Asia-Pacific Working Party and World Gastroenterology Organization (WGO)]. Using the ARGE II instrument, we found US 2012 and Europe 2016 had the highest scores, especially in the areas of rigor of development and applicability. Additionally, Italy 2010 and Korea 2013 also presented comprehensive content, rigorous procedures and good applicability. And WGO 2014 offered various algorithms for clinical practice. Lastly, a practical algorithm for the clinical management was developed, based on the recommended guidelines.

CONCLUSION

This is the first systematic review of NAFLD guidelines. It may yield insights for physicians and policy-makers in the development and application of guidelines.

Key words: Diagnosis; Management; Non-alcoholic fatty liver disease; Systematic review; Treatment

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Core tip: Non-alcoholic fatty liver disease (NAFLD) is one of the leading chronic liver diseases globally. A comprehensive study of NAFLD guidelines will be useful for various stakeholders to develop and utilize guidelines. This is the first systematic review to grade NAFLD guidelines and present recommendations for the clinical management of NAFLD. Through systematically evaluating the published guidelines and offering a clinical algorithm, it may yield insights for physicians and policy-makers in the development and application of guidelines.

Zhu JZ, Hollis-Hansen K, Wan XY, Fei SJ, Pang XL, Meng FD, Yu CH, Li YM. Clinical guidelines of non-alcoholic fatty liver disease: A systematic review. *World J Gastroenterol* 2016; 22(36): 8226-8233 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i36/8226.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i36.8226>

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of disease ranging from simple hepatic steatosis, to inflammatory non-alcoholic steatohepatitis (NASH) with increasing levels of fibrosis and eventually hepatic cirrhosis^[1]. According to the latest guideline released in Europe^[2], it is defined by the presence of steatosis in > 5% hepatocytes, in the absence of other causes attributed to hepatic steatosis^[3]. Recent advance supports NAFLD as the chronic liver disease component of metabolic syndrome^[4].

Younossi *et al*^[5] estimated the global prevalence of imaging-diagnostic NAFLD arrived at 25%, although it

varied by region and age. In the United States, it was reported to be between 10% and 30%, which is similar to rates in Europe and Asia^[6-8]. It is alarming that the prevalence of NAFLD worldwide is on the rise^[9], along with the associated disorders: obesity, insulin resistance, diabetes and metabolic syndrome^[8]. New evidence supports NAFLD as a common liver disease presenting across the globe, which warrants the attention of physicians, researchers, and national policy makers. However, gaps between provider knowledge and awareness of clinical practice guidelines exist.

Offering continuing education and developing high-quality national guidelines may help making inroads into the problem of suboptimal NAFLD care. A comprehensive study of the existing guidelines of NAFLD might be useful for helping stakeholders, including physicians, patients, policymakers and governmental bodies to develop and implement guidelines. To our knowledge, this is the first systematic critical appraisal of published guidelines to systematically grade and comprehensively present the evidence-based recommendations for the diagnosis and treatment of NAFLD.

MATERIALS AND METHODS

This systematic review was conducted according to the PRISMA guidelines^[10].

Electronic database search

The database search was conducted on PubMed for guidelines published before May 2016. In the search, we used the following key words and terms: ["fatty liver"(Title)] AND [strategy*(Title) OR guideline*(Title) OR recommendation*(Title) OR management*(Title)].

Websites searches

The literature search was supplemented by searching relevant websites (using the term "fatty liver"), including the following: (1) Australia National Health and Medical Research Council (<https://www.nhmrc.gov.au/?>); (2) American College of Physicians (<https://www.acponline.org/>); (3) American Medical Association (<http://www.ama-assn.org/ama>); (4) Institute for Clinical Systems Improvement (<https://www.icsi.org/>); (5) Institute of Medicine (<http://www.nationalacademies.org/>); (6) National Guidelines Clearinghouse (<https://www.guideline.gov/>); (7) National Institute for Health and Clinical Excellence (<https://www.nice.org.uk/>); (8) Royal College of Physicians (<https://www.rcplondon.ac.uk/>); (9) Scottish Intercollegiate Guidelines Network (<http://www.sign.ac.uk/>); and (10) World Health Organization (<http://www.who.int/en/>).

Inclusion criteria and guidelines selection

The guideline was included in this study, if it met the following criteria: (1) clinical guidelines regarding the diagnosis and management of NAFLD in adults; (2) released by governmental agencies or key health

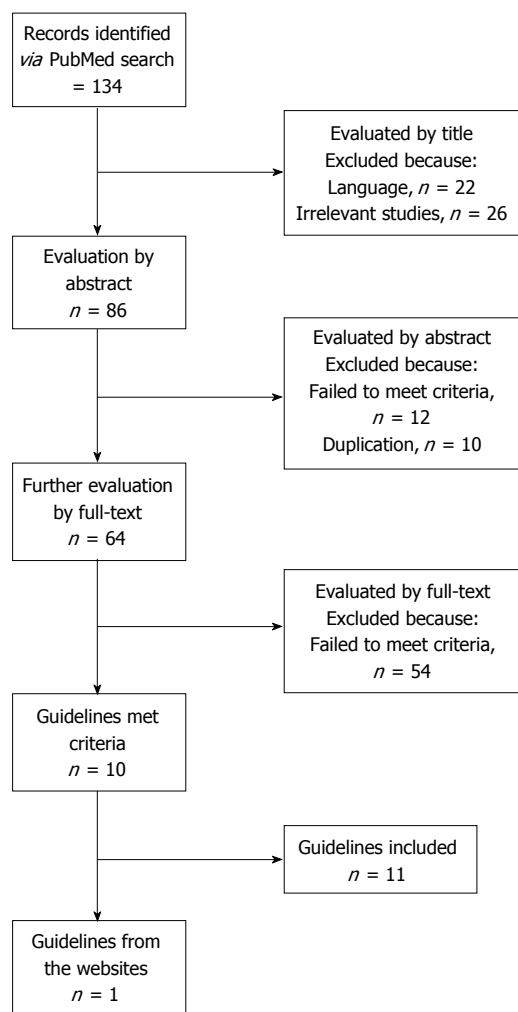


Figure 1 Flow chart of guidelines searching.

organizations; and (3) published in English. Two investigators independently performed the screen on PubMed and the websites, according to the inclusion criteria. Discrepancies were resolved by the involvement of a third reviewer.

The Appraisal of Guidelines for Research and Evaluation Instrument II

The Appraisal of Guidelines for Research and Evaluation (AGREE) II was a tool designed to appraise the methodological rigor and transparency in which a clinical guideline is developed and it is used internationally. It consists of 23 items grouped in 6 domains, *i.e.*, scope and purpose, stakeholder involvement, rigor of development, clarity and presentation, applicability and editorial independence^[11].

RESULTS

Guidelines included in the study

As shown in Figure 1, eleven guidelines met the criteria and were included in the final version of this systematic review. Since 2007, five guidelines were released in

Asia (3 in China, 1 in Japan and 1 in South Korea), while three guidelines were released in the United States, Italy and Chile, respectively (Table 1). Three guidelines were released by international agencies, *i.e.* Asia-Pacific Working Party, World Gastroenterology Organization (WGO) and a joint commission of European associations.

Guidelines quality scores

Eleven guidelines were appraised according to AGREE II, as presented in Table 1 and Figure 2. We highly recommended the two guidelines, United States 12^[1] and Europe 16^[2], given the high scores and the authority of the organizations. Additionally, Italy 10^[12] and South Korea 13^[13] also presented comprehensive content, rigorous procedures and good applicability. Lastly, WGO 14^[14] offered a variety of algorithms for clinical practice.

Clinical algorithm

Figure 3 presented a clinical algorithm for the diagnosis and management of NAFLD in adults. Generally, the procedure of clinical practice includes diagnosis, assessment, and management.

DISCUSSION

This is the first systematic critical appraisal to grade the guidelines and present the evidence-based recommendations for the clinical management of NAFLD. Using the ARGE II instrument, we found United States 12^[1] and Europe 16^[2] had the highest scores, especially in the areas of rigor of development and applicability. Additionally, we developed a clinical algorithm for the diagnosis and management of NAFLD in adults.

NAFLD is one of the leading chronic liver diseases in the world^[5]. While incidence rates may possibly vary and/or be underreported^[15,16], the present situation reinforces the need for a precise and rational system of management for NAFLD. Additionally, the obesity epidemic has led to a rapidly increasing population at risk for NAFLD, and shows no signs of slowing down. Therefore, NAFLD will only become a larger problem in the future if it is not properly prevented and managed now.

Currently, liver biopsy has still been regarded as the gold standard in the diagnostic evaluation of NAFLD^[17]. However, a biopsy is an invasive practice, which carries a series of medical risks, *e.g.* hemorrhage and infection^[2]. The non-invasive assessing method that is most suitable for evaluating hepatic steatosis is ultrasound, with a sensitivity of 60%-94% and a specificity of 66%-97%^[18], even though it presents less precise in milder degrees of steatosis. Given the widely availability and economic efficiency, ultrasound is recommended as a first-line diagnostic test in most guidelines, rather than liver biopsy and other

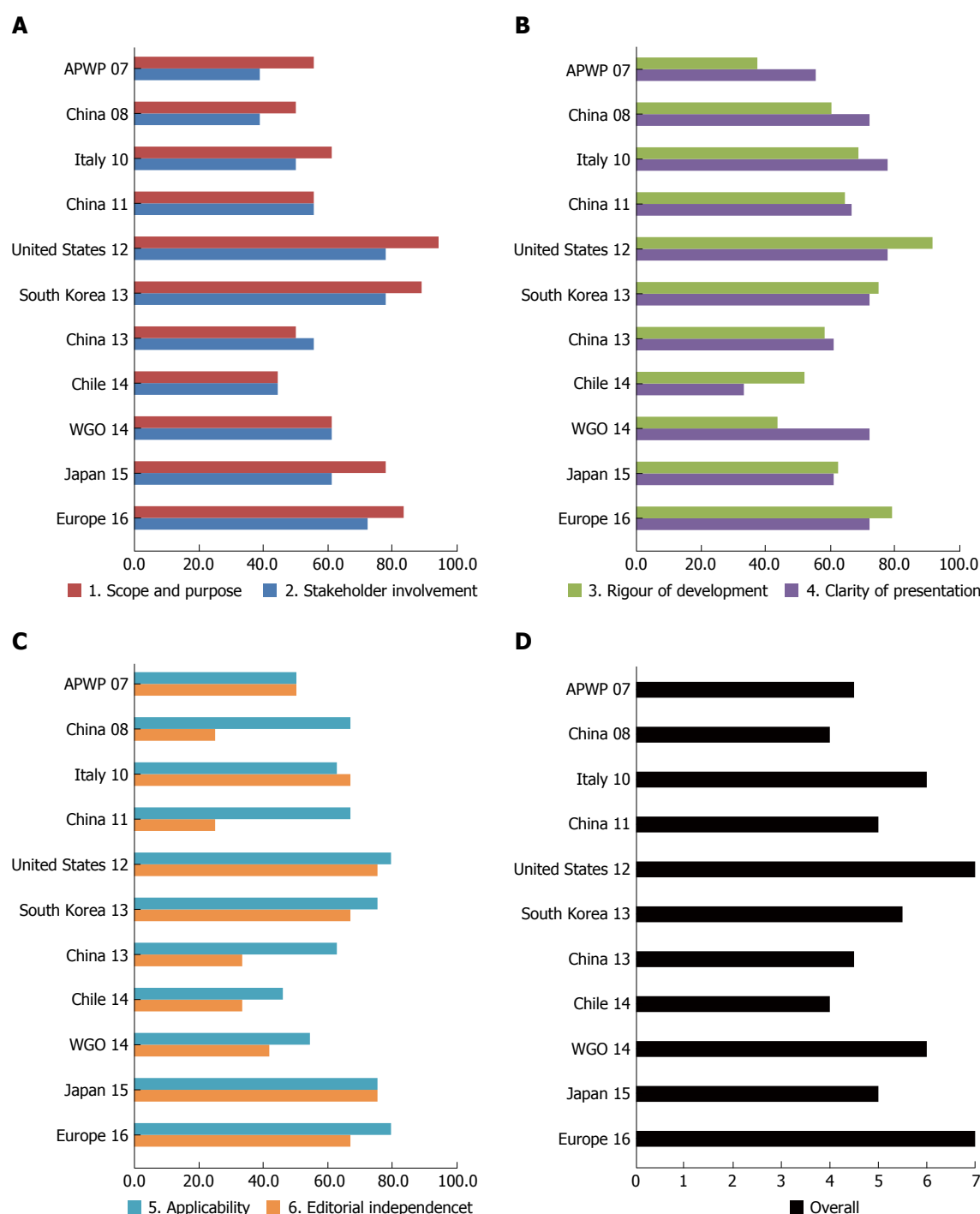


Figure 2 Domain scores for each guideline based on the Appraisal of Guidelines for Research and Evaluation II Instrument. A-C: Each Appraisal of Guidelines for Research and Evaluation II domain score for guidelines is presented on the X-axis as a percentage of 100 (0% = the domain was not at all satisfied; and 100% = fully satisfied). D: Overall scores for guidelines. WGO: World Gastroenterology Organization.

imaging tools. Additionally, a variety of noninvasive algorithms, based on metabolic and anthropometric tests, have been developed for identifying NAFLD, e.g. the fatty liver index^[19] and the hepatic steatosis index^[20]. They have been utilized to screen subjects with hepatic steatosis in large epidemiologic studies or predicting potential patients in clinical practice^[18]. The development of more accurate and noninvasive diagnostic tools is still a major unmet demand in the clinic.

In terms of treatment, the pathophysiological association between NAFLD and obesity-related diseases, e.g., metabolic syndrome and diabetes, supports structured programs of lifestyle intervention aimed at weight loss, before or in addition to pharmacotherapy^[3,21]. The elements of a comprehensive lifestyle approach generally include energy restriction, macronutrient composition, alcohol consumption, coffee drinking and physical activity^[2]. Furthermore, published guidelines suggested pharmacotherapy

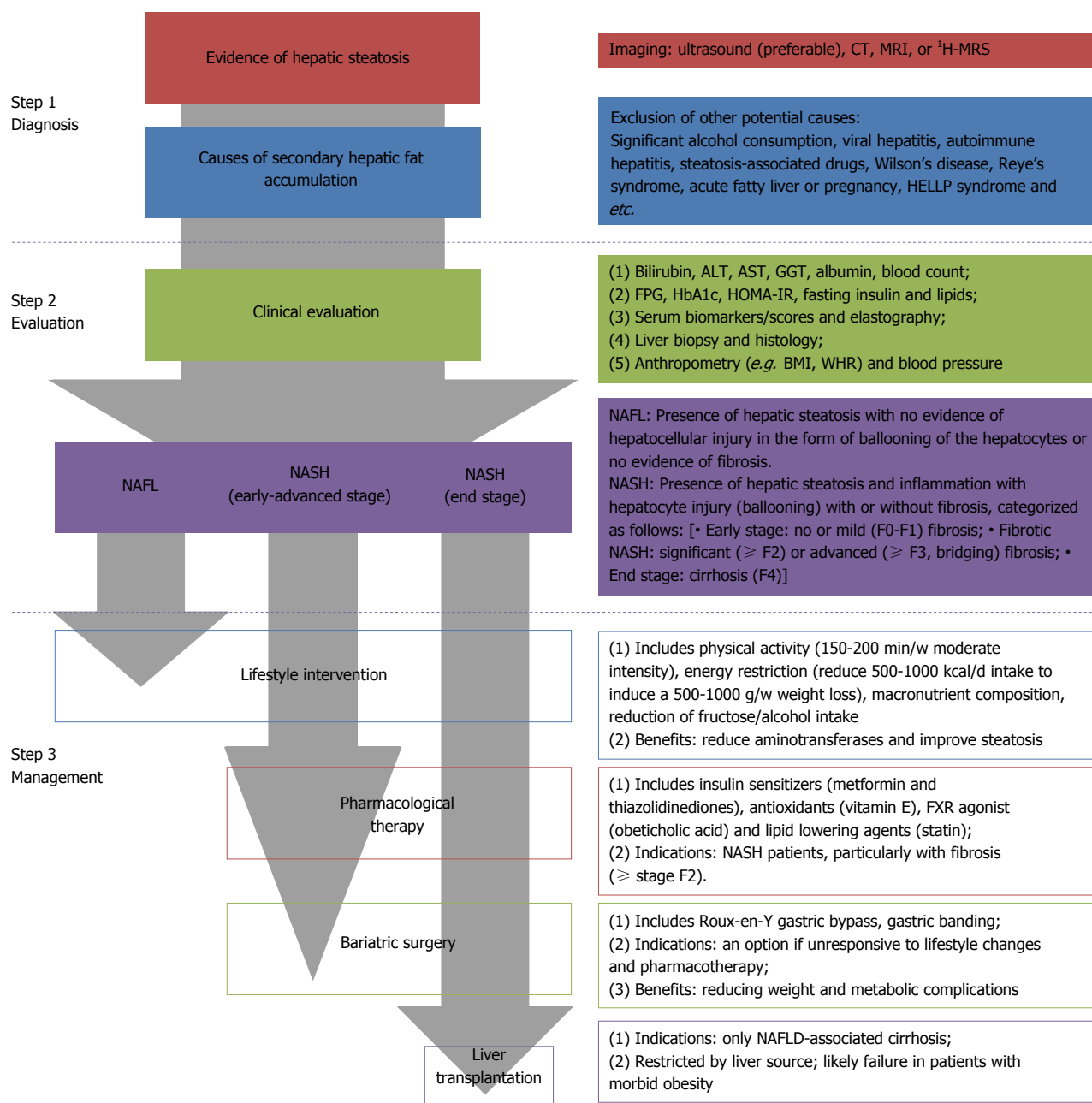


Figure 3 Clinical algorithm for the diagnosis and management of non-alcoholic fatty liver disease in adults. The algorithm was developed according to United States 12^[1], WGO 14^[13] and Europe 16^[2]. ¹H-MRS: Proton magnetic resonance spectroscopy; ALT: Alanine transaminase; AST: Aspartate transaminase; BMI: Body mass index; CT: Computed tomography; FPG: Fasting plasma glucose; FXR: Farnesoid X receptor; GGT: Gamma-glutamyltransferase; HbA1c: Hemoglobin a1c; HOMA-IR: Homeostasis model assessment of insulin resistance; MRI: Magnetic resonance imaging; WHR: Waist-to-hip ratio; WGO: World Gastroenterology Organization; NAFLD: Non-alcoholic fatty liver disease.

should be exclusively indicated for early-stage NASH with increased risk of advanced NASH^[2,22]. The past decade has witnessed some advance in clinical pharmacotherapy trials, *e.g.*, the use of metformin, pioglitazone and vitamin E, however most NASH patients failed to respond to these methods^[2,23]. When considering safety and tolerability, no drug has been approved for NAFLD by pharmacological agencies by now, while no specific drug therapy was firmly recommended in the present guidelines^[2]. Therefore, it is still imperative to continue research

to improve pharmacotherapy for NASH and hepatic fibrosis. Additionally, the role of bariatric surgery in the treatment of NAFLD is still unknown. Current evidence found that NAFLD patients who undergo bariatric surgery require long-term postoperative management, due to an increased risk for fibrosis progression^[1,14].

This is the first systematic review of published NAFLD guidelines. Using AGREE II^[11], it systematically grades the guidelines and presents the evidence-based recommendations for the clinical management of NAFLD. Additionally, a clinical algorithm for the

Table 1 Characteristics of non-alcoholic fatty liver disease guidelines included in this study

Author(s)/Organization(s)	Published Year	Region/country	Title	Recommendation
Chitturi <i>et al</i> ^[25] . Asia-Pacific Working Party on NAFLD (APWP 07)	2007	Asia-Pacific region	Non-alcoholic fatty liver disease in the Asia-Pacific region: Definitions and overview of proposed guidelines	Not recommended
Zeng <i>et al</i> ^[26] . The Chinese National Consensus Workshop on NAFLD (China 08)	2008	China	Guidelines for the diagnosis and treatment of nonalcoholic fatty liver diseases	Not recommended
Loria <i>et al</i> ^[12] . Italian Association for the Study of the Liver (Italy 10)	2010	Italy	Practice guidelines for the diagnosis and management of nonalcoholic fatty liver disease: A decalogue from the Italian Association for the Study of the Liver (AISF) Expert Committee	Recommended but modified
Fan <i>et al</i> ^[27] . Chinese Association for the Study of Liver Disease (China 11)	2011	China	Guidelines for the diagnosis and management of nonalcoholic fatty liver disease: Update 2010	Not recommended
Chalasani <i>et al</i> ^[1] . American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association (US 12)	2012	United States	The diagnosis and management of non-alcoholic fatty liver disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association	Recommended
The Korean Association for the Study of the Liver (South Korea 13) ^[13]	2013	South Korea	KASL clinical practice guidelines: Management of nonalcoholic fatty liver disease	Recommended but modified
Gao <i>et al</i> ^[28] . Study Group of Liver and Metabolism, Chinese Society of Endocrinology (China 13)	2013	China	Diagnosis and management of non-alcoholic fatty liver disease and related metabolic disorders: Consensus statement from the Study Group of Liver and Metabolism, Chinese Society of Endocrinology	Not recommended
Arab <i>et al</i> ^[29] . Chilean Society of Gastroenterology (Chile 14)	2014	Chile	Management of nonalcoholic fatty liver disease: An evidence-based clinical practice review	Not recommended
LaBrecque <i>et al</i> ^[14] . World Gastroenterology Organization (WGO 14)	2014	World	World Gastroenterology Organization global guidelines: Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis	Not recommended
Watanabe <i>et al</i> ^[30] . Japanese Society of Gastroenterology (Japan 15)	2015	Japan	Evidence-based clinical practice guidelines for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis	Not recommended
European Association for the Study of the Liver, European Association for the Study of Diabetes and European Association for the Study of Obesity (Europe 16) ^[2]	2016	Europe	EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease	Recommended

Ordered by publication year. Recommendation was developed based on AGREE II instrument. NAFLD: Non-alcoholic fatty liver disease.

clinical practice was developed, based on the highly recommended guidelines.

This study has some limitations. To begin with, only the guidelines in English were included in this review. Thus, high-quality guidelines in other languages might have been missed. Second, we chose the AGREE II instrument to evaluate the guidelines, even though there are other appraisals, *e.g.* Global Rating Scale^[24]. Third, guidelines should include information on how to reduce inappropriate practice and improve the efficiency of management. Further, the application of guidelines is crucial in clinical practice. However, we failed to evaluate the acceptance and the application of the guidelines in this review, due to the limited inclusion in the literature included in this review.

In this study, a systematic review was conducted to search and integrate the published guidelines of NAFLD. Furthermore, based on the evaluation of the included guidelines, a clinical algorithm for the diagnosis and management of NAFLD was developed. We hope it will yield insights for physicians and policy-makers in the development and application of guidelines moving forward.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is one of the leading chronic liver diseases globally. A comprehensive study of NAFLD guidelines will be useful for various stakeholders to develop and utilize guidelines.

Research frontiers

New evidence supports NAFLD as a common liver disease presenting across the globe, which warrants the attention of physicians, researchers, and national policy makers. However, gaps between provider knowledge and awareness of clinical practice guidelines exist. Offering continuing education and developing high-quality national guidelines may help making inroads into the problem of suboptimal NAFLD care.

Innovations and breakthrough

A comprehensive study of the existing guidelines of NAFLD might be useful for helping stakeholders, including physicians, patients, policymakers and governmental bodies to develop and implement guidelines. The authors think, this is the first systematic critical appraisal of published guidelines to systematically grade and comprehensively present the evidence-based recommendations for the diagnosis and treatment of NAFLD.

Applications

The systematic review included eleven published NAFLD guidelines in

the worldwide. Furthermore, we graded the guidelines, using the AGREE instrument II. Lastly, a practical algorithm for the clinical management was developed, based on the recommended guidelines.

Terminology

The Appraisal of Guidelines for Research and Evaluation Instrument II was a tool designed to appraise the methodological rigor and transparency in which a clinical guideline is developed and it is used internationally. It consists of 23 items grouped in 6 domains, *i.e.*, scope and purpose, stakeholder involvement, rigor of development, clarity and presentation, applicability and editorial independence.

Peer-review

The authors conducted the first systematic review of published NAFLD guidelines. It may yield insights for physicians and policy-makers in the development and application of guidelines.

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S- Editor: Gong ZM **L- Editor:** A **E- Editor:** Zhang FF



Diagnosis of colonic amebiasis and coexisting signet-ring cell carcinoma in intestinal biopsy

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Author contributions: Grosse A designed the report, collected the patient's clinical and histopathological data, analyzed and interpreted the data and wrote the paper.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board (Kantonale Ethikkommission Zürich).

Informed consent statement: Written informed consent was obtained from the patient for publication of this Case Report and any accompanying images.

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Abstract

Amebiasis is uncommon in developed countries. Several case reports in the literature emphasize that both the presenting symptoms and the radiological findings of colonic amebiasis closely resemble more common conditions, such as idiopathic inflammatory bowel disease and gastro-intestinal malignancy. We describe a unique case of colonic amebiasis (amebomas) coexisting with signet-ring cell carcinoma of the ileocecal valve, the cecum and the appendix. Endoscopically, the ulcerated tumor was indistinguishable from the ulcerations and pseudotumors (amebomas) detected in the ascending colon. Histological examination of biopsy specimens revealed the pathognomonic features of protozoa with ingested erythrocytes in combination with signet-ring cell infiltration. The author concludes that amebiasis may not only mimic carcinoma but, rarely, may coexist with carcinoma in the same patient. Clinicians and pathologists should be aware of this possibility in order not to delay diagnosis and treatment of malignant disease.

Key words: Colonic amebiasis; Histopathology; Parasitic disease; Colorectal carcinoma; Colonoscopy

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Core tip: This case report presents the unique case of a patient in whom colonic amebiasis (amebomas) and signet-ring cell carcinoma of the ileocecal valve, the cecum and the appendix were diagnosed in intestinal biopsy specimens. In the setting of amebiasis, malignancy may easily be overlooked. Therefore, clinicians and pathologists should be aware that colonic amebiasis and colorectal carcinoma may coexist in the same patient.

Grosse A. Diagnosis of colonic amebiasis and coexisting signet-ring cell carcinoma in intestinal biopsy. *World J Gastroenterol* 2016; 22(36): 8234-8241 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i36/8234.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i36.8234>

INTRODUCTION

Amebiasis caused by *Entamoeba histolytica* (*E. histolytica*) is one of the most fatal parasitic diseases worldwide. The organism is commonly acquired by ingestion of contaminated food and water and is endemic in developing countries with poor sanitation. Presentation of intestinal disease ranges from asymptomatic infection or acute proctocolitis to acute fulminant colitis with high mortality rates. Both the clinical presentation and the endoscopic appearance of colonic amebiasis can mimic colon carcinoma^[1-8]. We present an exceedingly rare case of colonic amebiasis (amebomas) with coexisting signet-ring cell carcinoma of the ileocecal valve, the cecum and the appendix diagnosed upon histological and immunohistochemical examination of intestinal biopsy specimens.

CASE REPORT

A 35-year-old woman presented to our hospital with diarrhea, right-lower-quadrant abdominal pain and cramps of two months' duration. The patient gave a travel history to Nepal where she had studied for one year prior to presentation. Her medical history was otherwise unremarkable; in particular, she had no family history of chronic idiopathic inflammatory bowel disease.

Laboratory tests revealed normocytic anemia (hemoglobin 105 g/L, hematocrit 0.34 L/L, and erythrocytes 3.70 T/L), with low vitamin B-12 (130 ng/L), high serum iron (35.3 µmol/L), significantly increased serum ferritin (1042 µg/L) and normal transferrin, as well as neutrophilic leukocytosis (leukocytes 11.52 G/L and neutrophils 9.30 G/L). Magnetic resonance imaging (MRI) showed mural thickening of the ileocecum and the appendix, which was interpreted as being inflammatory in nature (Figure 1); there was no evidence of an abscess. MRI also disclosed a nonhomogeneous, cystic mass of the left ovary that measured 7.9 cm in diameter. Gastroscopy and proctoscopy including anorectal examination were inconspicuous. Colonoscopy revealed multiple ulcerations and up to 10 well-circumscribed, ulcerated, tumor-like masses, separated by normal mucosa, in the ascending colon (Figure 2A and B). The masses were livid-colored at the periphery and necrotic at the center, and these masses ranged in size from 8 mm to 33 mm in diameter. A similar, but larger (4.5

cm in diameter) and more solid lesion was found at the ileocecal valve involving the adjacent cecum and the appendix (Figure 2C and D). These endoscopic manifestations were considered to be inconclusive. While the ulcerated lesions were found to be atypical of Crohn's disease, differential diagnoses included tuberculosis, lymphoma and carcinoma.

Microscopically, the masses in the ascending colon consisted of necrosis with large amounts of cell debris, desquamated epithelial cells and neutrophils, and extensive inflammatory tissue reaction in the absence of malignancy. Both in the debris and in the submucosal tissues there was evidence of periodic acid-Schiff (PAS)-positive amebas that displayed characteristic features of the invasive pathogen with ingested erythrocytes (Figure 3A-D). Because of the absence of malignancy, the masses in the ascending colon were considered to be amebomas, a complication of chronic amebic disease. Microscopic examination (hematoxylin-eosin and immunohistochemical stains) of biopsy specimens from the ileocecal valve showed amebas intermixed with signet-ring cells infiltrating the submucosa (Figure 3E-J). The CK 7, CK 20 and CDX-2 positive tumor cells, which stained negative with neuroendocrine markers, demonstrated preserved expression of the mismatch-repair proteins MLH1, PMS2, MSH2 and MSH6, indicating a microsatellite stable phenotype of carcinoma.

The pre-operative patient work-up included computed tomographic imaging of the chest and upper abdominal region, which revealed no extraintestinal manifestations of amebiasis, and explorative laparoscopy (Figure 4A and B) along with cytological examination of and biopsies from the peritoneum, which showed peritoneal carcinomatosis. After treatment with metronidazole, the patient underwent chemotherapy. Four months after diagnosis, right-sided hemicolectomy, omentectomy and Douglas-resection with hysterectomy and bilateral salpingo-oophorectomy were performed. Gross pathology of the surgical specimens showed tumor involvement of the cecum, the appendix and the ileocecal valve (Figure 5A). Metastases were found in the regional lymph nodes, the ovaries (Figure 5B), the fallopian tubes, the peritoneum, the omentum and the diaphragm. The biopsy-based diagnosis of signet-ring cell adenocarcinoma was confirmed by histology of the resected specimens (Figure 6A-F).

DISCUSSION

E. histolytica is a major cause of diarrhea in developing countries, primarily in tropical and subtropical regions with poor sanitation and inadequate barriers between food/water and human feces. In developed countries, most infections arise in immigrants and travelers

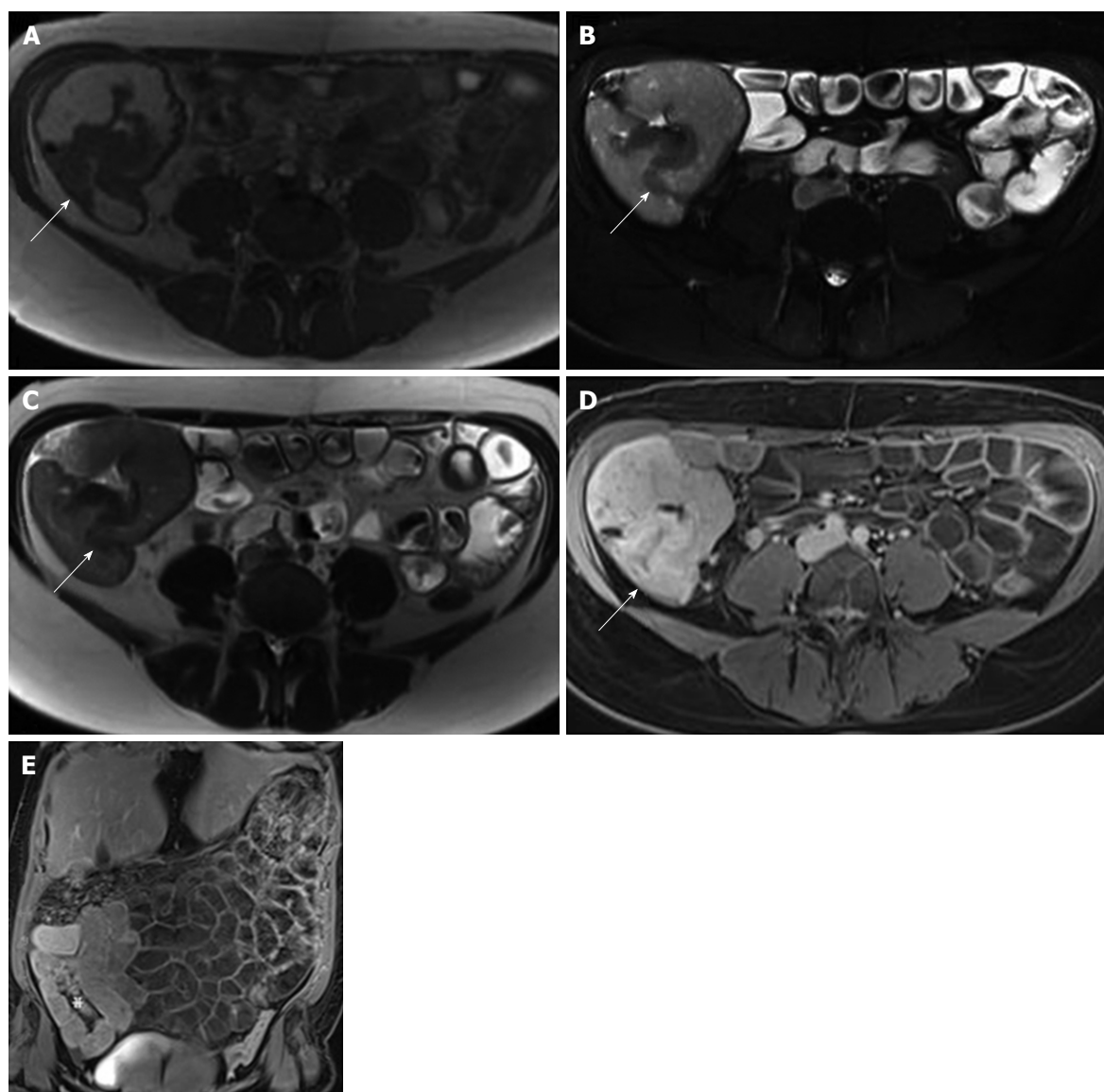


Figure 1 Magnetic resonance imaging showed mural thickening of the ileocecum and the appendix, which was interpreted as being inflammatory in nature. A-E: Magnetic resonance images (A: T2-weighted magnetization transfer contrast transversal; B: T2-weighted fat saturated transversal; C: T2-weighted half-Fourier acquired single-shot turbo spin-echo transversal; D: T1-weighted VIBE-Dixon postcontrast transversal, E: T1-weighted VIBE-Dixon postcontrast coronal) showing mural thickening (arrows in A-C) of the ileocecum and the appendix with contrast enhancement (arrow in D, asterisk in E).

from endemic areas. Young infants, pregnant women, malnourished individuals, and immunocompromised individuals are at highest risk for severe disease, which is characterized by complications such as perforation, toxic megacolon, fistulae, liver abscess and hemorrhage. Each year *E. histolytica* causes 40-50 million symptomatic infections worldwide, and approximately 40000 to 100000 people die each year from the disease, making this condition the second leading cause of death among parasitic diseases worldwide (after malaria)^[9-11]. The majority of the patients (90%) remain asymptomatic^[12,13]. Symptomatic patients can have simple acute proctocolitis or develop uncommon forms of GI involvement that include toxic megacolon, which is usually associated with corticosteroid treatment, abscess formation,

and acute fulminant colitis with a mortality rate over 40%^[14]. Clinical symptoms range from mild, with diarrhea, abdominal cramps and right lower quadrant tenderness ("non-dysenteric" infection), to severe, with abdominal cramps, fever and mucoid or bloody diarrhea ("dysenteric or invasive" infection).

The infestation begins with ingestion of *E. histolytica* cysts from food or water contaminated with feces. After digestion of the cysts in the intestinal lumen trophozoites are released. The trophozoites reproduce by clonal expansion and finally form cysts in the colon that are excreted in the feces and into the environment completing the life cycle of the parasite. *E. histolytica* has phagocytic, proteolytic and cytolytic capabilities and is known to secrete metabolic enzymes that facilitate their invasion into the mucosa and submucosa

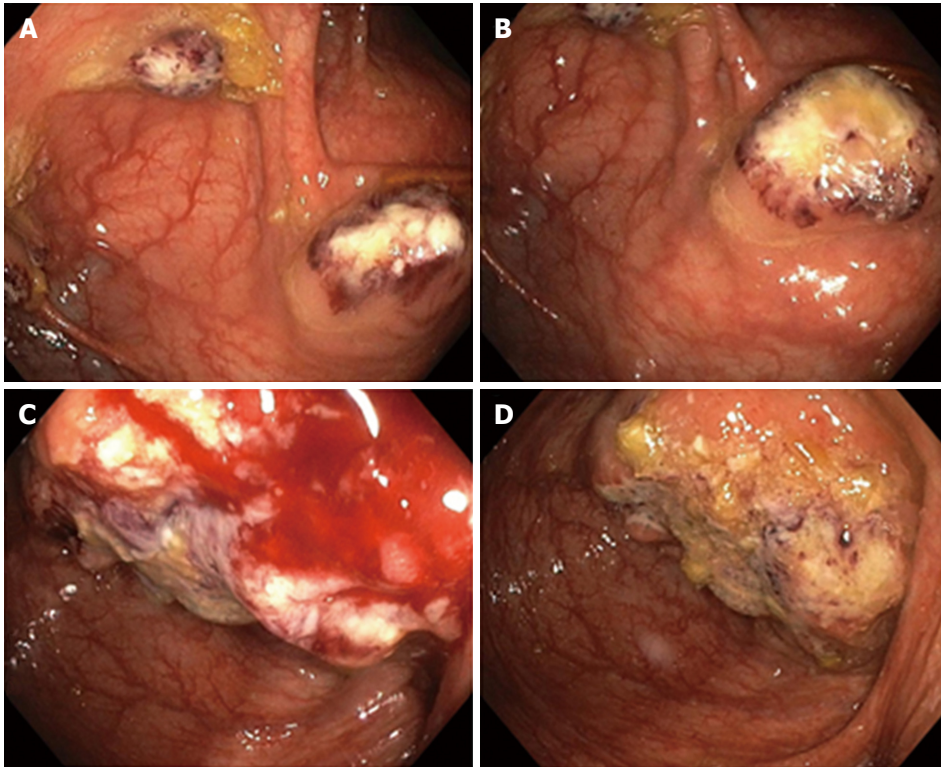


Figure 2 Colonoscopy. A, B: Colonoscopy showing multiple pseudotumors (amebomas) in the ascending colon, mimicking carcinoma; C, D: Endoscopic view of a large ulcerated tumor mass at the ileocecal valve.

causing characteristic flask-shaped intestinal ulcers. The parasites may penetrate through the mucosa and submucosa into the portal circulation and reach internal organs such as the liver, lungs, skin, etc. Amebiasis may involve any part of the bowel, but the cecum and the ascending colon are predilection sites^[9]. The most common extraintestinal manifestation is liver abscess caused by hematogenous spread from the GI tract.

The pathophysiological mechanism includes adhesion of *E. histolytica* to the intestinal epithelial cells via the Gal/GalNAc lectin (galactose/N-acetylgalactosamine specific lectin) triggering host cell death through phagocytosis, cytotoxicity and caspase activation. Other molecules involved in the disease process are amebapores, a serine-rich *E. histolytica* protein, and cysteine proteases^[15,16]. The ulcers observed on endoscopy vary in size from pin-head size to large (> 2.5 cm in diameter), coalescing, geographic or serpiginous lesions with intervening normal mucosa. Amebomas, a late complication of the disease, are pseudo-tumoral lesions formed by granulation tissue with peripheral fibrosis and a core of chronic inflammation usually found in the cecum or ascending colon. Amebomas may cause obstructive symptoms and can be confused with malignancy. In addition, both the endoscopic appearance and the histologic features (including crypt architectural distortion, mural fibrosis and scarring) of chronic amebic colitis may resemble

Crohn's disease. In contrast to amebic colitis, Crohn's disease does not spare the upper gastrointestinal tract and shows additional histological features typical of chronic idiopathic inflammatory bowel disease, such as granulomas, crypt abscesses and transmural lymphoid aggregates in the absence of protozoa.

Although a number of antigenic and molecular diagnostic tools have been developed over the years^[17], the most commonly used method for diagnosis of intestinal amebiasis remains the examination of stool or intestinal biopsy by microscopy. The reported sensitivity of microscopic stool examination in identifying amebic protozoa ranges from 25% to 60%^[18]. Histological features of *E. histolytica* include distinct cell membranes, foamy cytoplasm, round and eccentrically located nuclei with margination of nuclear chromatin, and ingested red blood cells. The parasites may resemble macrophages but are CD-68 negative, PAS and trichrome positive and have smaller nuclei. Because of the conversion of glucose and pyruvate to ethanol, these organisms are highly motile, resulting in a pleomorphic shape (with diameters ranging from 10 to 50 μm). In the current case, the diagnosis of colonic amebiasis and signet-ring cell carcinoma was established by histological examination of intestinal biopsy specimens showing the pathognomonic features of protozoa with ingested erythrocytes in combination with infiltrating tumor cells. While the findings were consistent with *E. histolytica* amebomas, antigenic or molecular tests for definitive

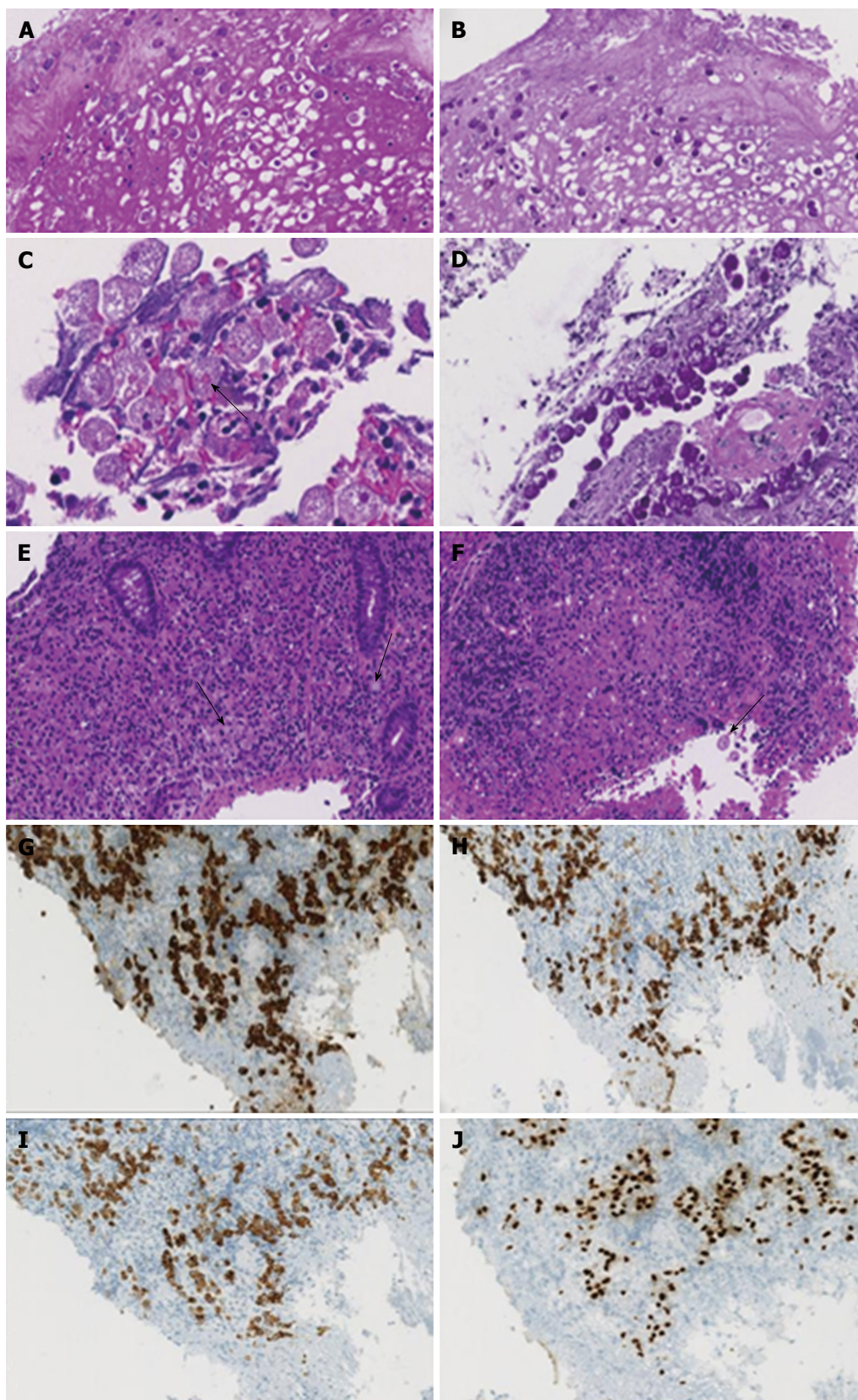


Figure 3 Microscopic examination. A, B: Microscopy (A: HE stain, $\times 20$, B: PAS, $\times 20$) of biopsy specimens from the ascending colon showing aggregations of round PAS-positive *Entamoeba histolytica* trophozoites within cell debris; C, D: High power view (C: HE stain, $\times 40$, D: PAS, $\times 20$) demonstrating PAS-positive trophozoites containing ingested erythrocytes (arrow in C); E, F: Microscopy (HE stain, $\times 20$) of biopsy specimens from the ileocecal valve showing infiltrating signet-ring cells (arrows in E) in combination with amebic protozoa (arrow in F); G-J: The tumor cells stain positive with pancytokeratin (G: $\times 20$), CK 7 (H: $\times 20$), CK 20 (I: $\times 20$) and CDX-2 (J: $\times 20$).

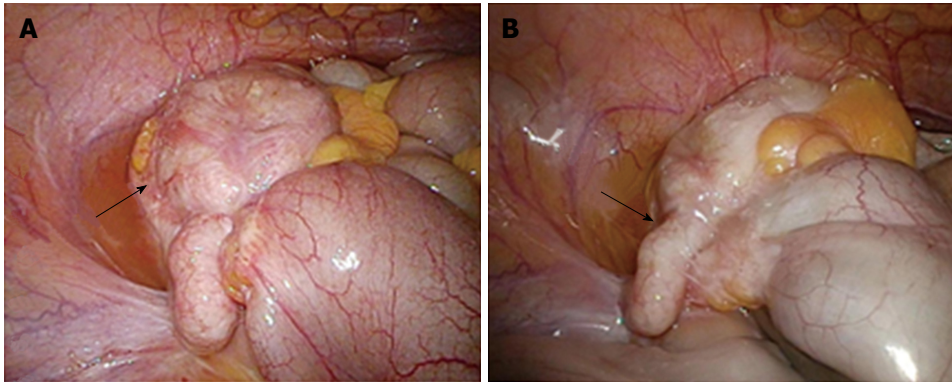


Figure 4 Explorative laparoscopy. A, B: Laparoscopic view of a tumor mass (arrows) at the ileocecum and the appendix.

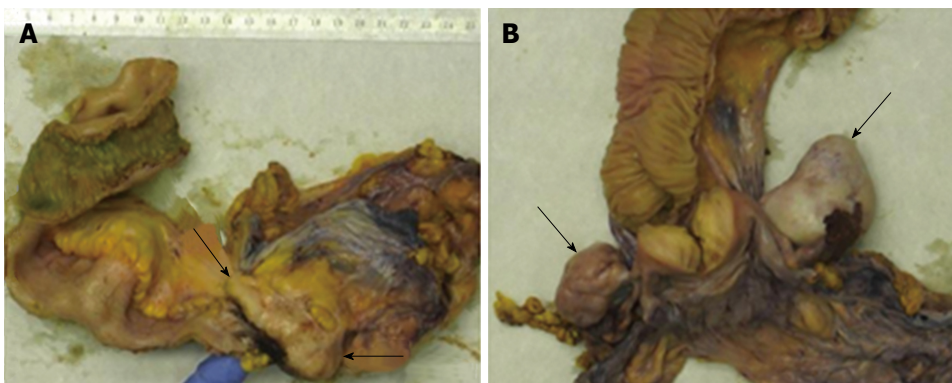


Figure 5 Gross pathology of the surgical specimens. A: Gross image showing a tumor mass (arrows) that involves the appendix, the ileocecal valve and the cecum. Surgical resection was performed after eradication of amebiasis and chemotherapy. B: Gross image of the Douglas-resection showing bilateral ovary metastases (arrows).

species identification were not performed, posing a limitation to the current study. Thus, (co-)infection with other morphologically indistinguishable *E. species* (*E. dispar* and *E. moshkovskii*) cannot definitively be excluded. Several investigations provide evidence that the prevalence of *E. histolytica*, derived from studies using conventional diagnostic tools (microscopy and/or culture), is often overestimated because of its epidemiological overlap with non-pathogenic *E. species*, and only a proportion of microscopically positive stool samples are true *E. histolytica* infections as confirmed by polymerase chain reaction assay^[19-22]. However, given that *E. dispar* is generally considered non-invasive and thus related to asymptomatic infections (despite emerging evidence that *E. dispar* can acquire pathogenicity in the presence of bacteria and produce significant lesions in the human intestine and liver^[23]), based on the clinical presentation, *E. histolytica* is likely the causative agent of dysenteric colitis and amebomas in the current case.

Colonic amebiasis can mimic colon carcinoma clinically, radiologically and endoscopically^[1-8]. Conversely, amebiasis and coexisting carcinoma are exceedingly rare^[24-29]. In two African studies, colorectal carcinoma was found to be associated with intestinal amebiasis in 6.1% and 6.5% of cases^[24,25]. Furthermore, five cases of cervical, perineal, sigmoid and pulmonary carcinomas colonized by *E. histolytica* have been described in three case reports^[26-28]. However, apart from a single case study dated from 1963^[29], to our knowledge the unique coincidence of colon carcinoma and amebomas has not been published previously in the literature.

The unusual co-occurrence of amebic infection and carcinoma poses particular challenges for both the pathologist and the clinician. Because coexisting malignancy in the setting of amebic disease can easily be overlooked, careful evaluation of individuals with amebiasis is necessary to confirm or rule out accompanying malignant disease.

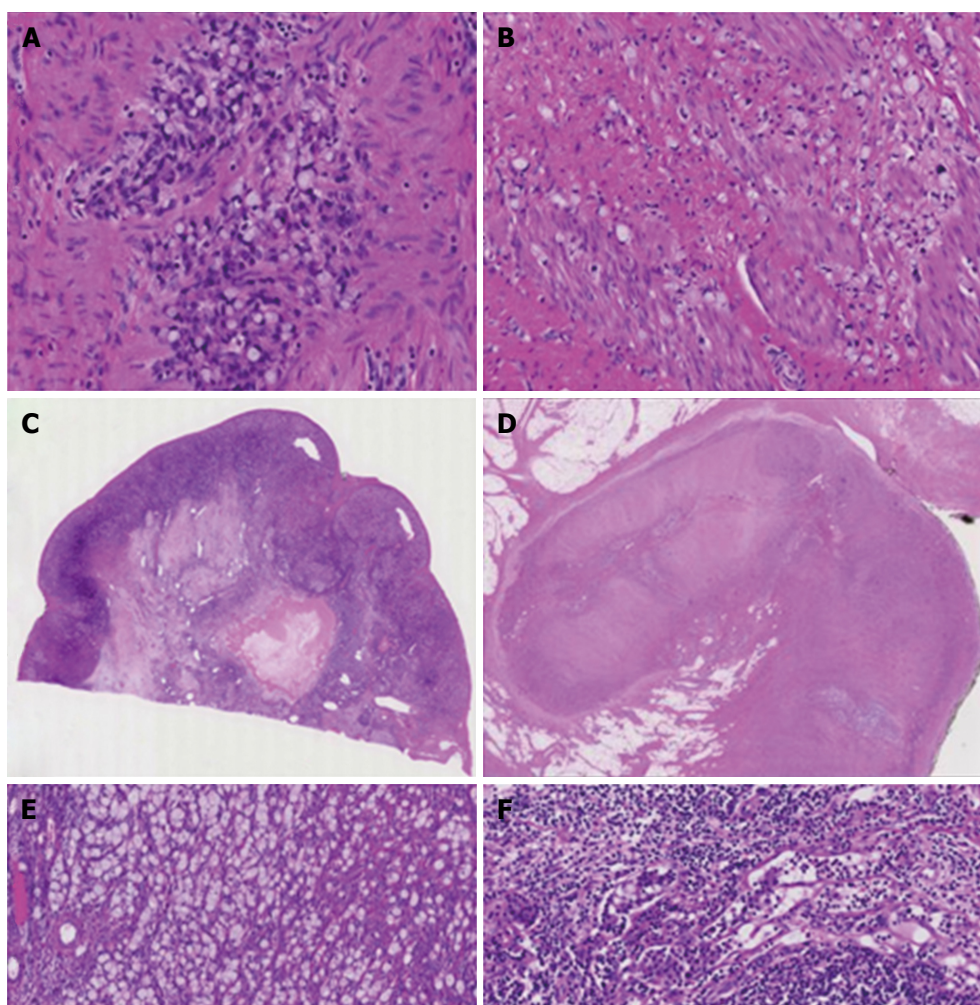


Figure 6 Biopsy-based diagnosis of signet-ring cell adenocarcinoma was confirmed by histology of the resected specimens. A, B: Microscopy (A: HE stain, $\times 40$, B: HE stain, $\times 20$) of surgical specimens demonstrating infiltration of the colonic wall by signet-ring cells; C, D: The tumor involves the cecum (C: HE stain, $\times 4$) and the appendix (D: HE stain, $\times 4$); E, F: Microscopy showing metastases to the ovary (D: HE stain, $\times 10$) and lymph nodes (E: HE stain, $\times 10$).

COMMENTS

Case characteristics

A 35-year-old woman with no significant medical history, but a travel history to Nepal, presented with diarrhea and right-lower-quadrant abdominal pain and cramps of two months' duration.

Clinical diagnosis

Right-lower-quadrant tenderness and mucoid diarrhea.

Differential diagnosis

Carcinoma, lymphoma, tuberculosis or Crohn's disease.

Laboratory diagnosis

Laboratory tests showed neutrophilic leukocytosis and normocytic anemia, thought to be related to vitamin B-12 deficiency and occult intestinal bleeding.

Imaging diagnosis

Magnetic resonance imaging showed mural thickening of the cecum, appendix and adjacent terminal ileum. Colonoscopy disclosed multiple ulcerated, tumor-like masses (up to 33 mm in diameter) in the ascending colon and a 4.5 cm solid lesion at the ileocecal valve. Computed tomographic imaging of the chest

and upper abdominal region revealed no extraintestinal mass-like lesions.

Pathological diagnosis

Amebomas and coexisting signet-ring cell carcinoma of the ileocecal valve, the cecum and the appendix with metastatic spread to the regional lymph nodes, ovaries, fallopian tubes, peritoneum, omentum and diaphragm.

Treatment

Treatment with metronidazole followed by chemotherapy and right-sided hemicolectomy, omentectomy, hysterectomy and bilateral salpingo-oophorectomy.

Related reports

Several case reports emphasize that colonic amebiasis can mimic colon carcinoma leading to misdiagnosis. Rarely, colorectal carcinoma may be colonized by *E. dispar* and *E. moshkovskii*. Apart from a single case report, the unique coincidence of colon carcinoma and amebomas has not been published previously in the literature.

Term explanation

Amebomas, a late manifestation of amebiasis, are pseudo-tumoral lesions. They consist of granulation tissue with a core of chronic inflammation and

typically occur in the cecum or ascending colon.

Experiences and lessons

Colonic amebiasis can be confused with a neoplastic process. However, rarely, colon carcinoma and amebic disease may coexist in the same patient. Gastroenterologists and pathologists should be aware of this possibility and carefully evaluate patients with amebiasis to rule out concurrent malignancy.

Peer-review

The paper is well written. A limitation of the current study lies in the fact that definitive species identification was not performed using antigenic or molecular methods. However, the clinical presentation of the patient indicates that *Entamoeba histolytica* is likely the causative agent of dysenteric colitis and amebomas in the current case.

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Case of a tumor comprising gastric cancer and duodenal neuroendocrine tumor

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Author contributions: Kaneko H performed the majority of experiments and wrote the manuscript; Miyake A was in charge of the pathological diagnosis; Ishii Y, Sue S, Miwa H, Sasaki T, Tamura T and Kondo M participated to the medical care of the patient; Maeda S served as scientific advisor and participated to the medical care of the patient.

Institutional review board statement: This case report was approved by the ethics committee of Yokohama City University Graduate School of Medicine.

Informed consent statement: All study participants provided informed written consent prior to the treatment.

Conflict-of-interest statement: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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Abstract

The present report describes a rare case of a tumor composed of early gastric cancer and a duodenal neuroendocrine tumor (NET). A 78-year-old woman underwent esophagogastroduodenoscopy at a local institution for screening of the upper gastrointestinal tract which revealed a protruded tumor through the pyloric ring from the pyloric antrum. The tumor was too large to treat at the facility; consequently, she was referred to our hospital for further management. Esophagogastroduodenoscopy with tumor biopsy of the lesion revealed the diagnosis of early gastric cancer. Endoscopic submucosal dissection was performed with sufficient free margins in both vertical and horizontal directions. Histopathological findings showed NET confined to the submucosal layer and covered by well-differentiated adenocarcinoma. Immunohistochemical stainings showed that the two lesions existed continuously. While the possibility of a collision cancer was considered, it was suggested that the two lesions existed continuously. Finally, the tumor was diagnosed as gastric cancer composed of duodenal NET G1, with a lymphatic invasion of NET component.

Key words: Gastric cancer; Endoscopic submucosal dissection; Neuroendocrine tumor; Composite- type tumor; Duodenum

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Core tip: Although there are some reports of endoscopic resection performed to duodenal neuroendocrine tumor (NET), no reports have been published on the treatment of duodenal NET composed of early gastric cancer treated by endoscopic submucosal dissection (ESD). A 78-year-old woman underwent esophagogastroduodenoscopy and revealed a protruded tumor through the pyloric ring from the pyloric antrum. ESD was performed and finally the tumor was diagnosed as gastric cancer composed of duodenal NET G1, with a lymphatic invasion of NET component. We report the first case of early gastric cancer accompanied by duodenal NET, which was resected by ESD.

Kaneko H, Miyake A, Ishii Y, Sue S, Miwa H, Sasaki T, Tamura T, Kondo M, Maeda S. Case of a tumor comprising gastric cancer and duodenal neuroendocrine tumor. *World J Gastroenterol* 2016; 22(36): 8242-8246 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i36/8242.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i36.8242>

INTRODUCTION

Endoscopic submucosal dissection (ESD) is widely performed for gastric cancer, and is also used for other cancers, such as esophagus, colon, rectum. The use of ESD for treatment for superficial non-ampullary duodenal epithelial tumors is also increasing^[1].

Duodenal neuroendocrine tumor (NET) is a rarely encountered tumor, with fewer cases reported than those for gastric and rectal carcinoid tumors, and its natural history has not yet been well defined^[2]. Although there are some reports of endoscopic resection, no reports have been published on the treatment of duodenal NET composed of early gastric cancer treated by ESD. We here report the first case of early gastric cancer accompanied by duodenal NET resected by ESD.

CASE REPORT

A 78-year-old woman presented to a local institution with nausea and right hypochondrial pain. Careful examination led to the diagnosis of gallstones and common bile duct stones. Esophagogastroduodenoscopy (EGD) screening of the upper gastrointestinal tract was performed before surgery to remove the gallstones and a protruded tumor through the pyloric ring from the pyloric antrum was found. Although biopsy specimen from the lesion was diagnosed as an adenoma, the tumor was too large to treat at the facility. The patient was referred to our hospital for further management of the gastric tumor. She had no specific medical history except hypertension and dyslipidemia.

No abnormalities were found on physical examination, including vital signs, and routine laboratory analyses. Serum levels of tumor markers such as carcinoembryonic antigen and carbohydrate antigen 19-9 were 2.9 ng/mL and 9 U/mL, respectively. Computed tomography of the abdomen revealed no mass in the gastrointestinal tract and no lymph node swelling or metastasis. EGD revealed a protruded tumor with a diameter of approximately 20 mm over the bulb from the pylorus antrum (Figure 1A and B). Magnifying endoscopy with narrow-band imaging showed irregularities in the structures and vessels of the tumor, which led to the suspicion of early gastric cancer (Figure 1C). Biopsy from the gastric portion of the tumor revealed well-differentiated adenocarcinoma. We were unable to investigate the whole tumor because it occupied and existed beyond the pyloric ring; however, there were no obvious signs suggesting submucosal tumor invasion. Although the tumor extended to the duodenum, the major part of the tumor was from the stomach. Finally, the diagnosis was early gastric cancer, and ESD was recommended.

The tumor was resected in a single fashion by the ESD technique, using a GIF-Q260J instrument (Olympus, Tokyo, Japan) with a transparent hood (MAJ-1989-11302; Olympus) attached to the tip of the gastroscope, a dual knife (Olympus), and an insulated tipped knife 2 (Olympus). An electrosurgical current generator (VIO200; ERBE, Tübingen, Germany) was used for the ESD. The tumor was resected with no complications, such as perforation or delayed bleeding. As a result, the patient was discharged on postoperative day 5.

Figure 2 shows a macroscopic view of the resected specimen. The resected mucosa and the tumor measured 54 mm × 40 mm and 38 mm × 32 mm, respectively. The histopathology results showed the resected specimen had sufficient free margins in both vertical and horizontal directions, but the tumor consisted of two components: The first part with irregular atypical epithelium, which formed a tubular and papillary structure confined to the mucosal layer, was a well differentiated adenocarcinoma, and the second part was located near the edge of duodenum side of the specimen and showed a mass composed of nests of small uniform tumor cells, which is typical of NET (Figure 3A-C). These cells were positive for CD56 (Figure 3D), synaptophysin (Figure 3E), and chromogranin A (Figure 3F), and the Ki-67 labeling index was less than 1%. According to the WHO 2010 classification, obvious nuclear fission images were not admitted and finally the NET was diagnosed as G1^[3]. The NET cells were detected from the mucosal layer over the submucosa, but no infiltration of the muscle layer was recognized.

The adenocarcinoma cells and the NET cells were in contact. Although migration images were not detected, both component cells had mixed in some parts and formed a tumor duct. Immunohistochemical staining

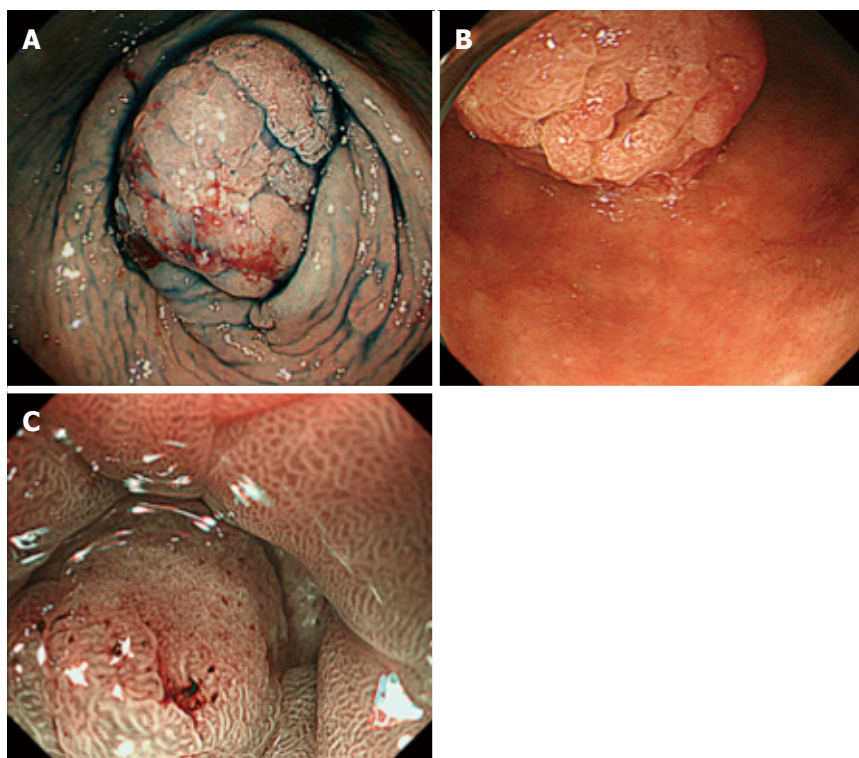


Figure 1 Esophagogastroduodenoscopic views of the tumor in the stomach. A: The protruded tumor occupied and existed beyond the pyloric ring, the whole tumor could not fit in one field of view; B: Image observed by inverting the endoscope in the duodenal bulb; C: Magnifying endoscopy with narrow-band imaging showed structure irregularities compared with the normal surrounding mucosa.

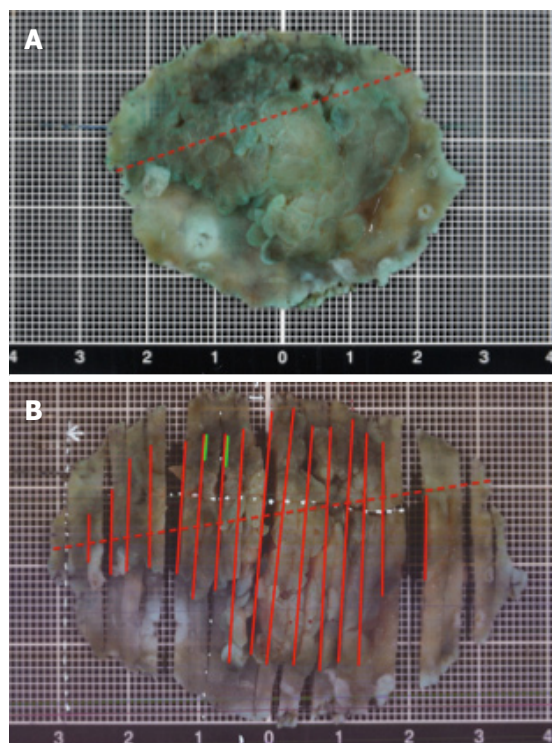


Figure 2 A macroscopic view of the specimen resected by endoscopic submucosal dissection. A: Macroscopic appearance of the specimen soaked for almost 24 h in formalin after endoscopic submucosal dissection. The red dotted line indicates where there was a pyloric ring; B: Cut out of the resected specimen. The solid red line indicates adenocarcinoma; green line indicates neuroendocrine tumor; red dotted line indicates the location of the pylorus.

also showed NET cells coexisting with adenocarcinoma cells (Figure 3E and F). No venous invasion was revealed by Elastica van Gieson staining, however, lymphatic invasion of NET component was seen with D2-40 staining (Figure 3G).

The patient was sufficiently explained about the results of the histopathological examination and the risk of lymph node metastasis; she refused additional operation and opted for careful observation. Therefore, close follow-up has been scheduled.

DISCUSSION

Since Oberndorfer proposed the term “carcinoid” in 1907, the origins of NET of the gastrointestinal tract, as well as the malignancy of these tumors, have been attracting the attention of clinicians^[3]. NETs are quite rare and almost 55% of NETs occur in the gastrointestinal tract^[2]. In Japan, NET occurs more frequently in the rectum and the stomach followed by the duodenum, and primary NET of the duodenum may occur in less than 5% of all cases^[2,4].

Although there were some reports about gastric collision tumor composed of epithelial and nonepithelial malignant neoplasm, Morishita *et al*^[5] reported that a simultaneous incidence of adenocarcinoma and malignant lymphoma was the most frequent finding and cases of gastric collision tumor composed of adenocarcinoma and NET were rare.

Previously, Kato *et al*^[6] reported a case of duodenal

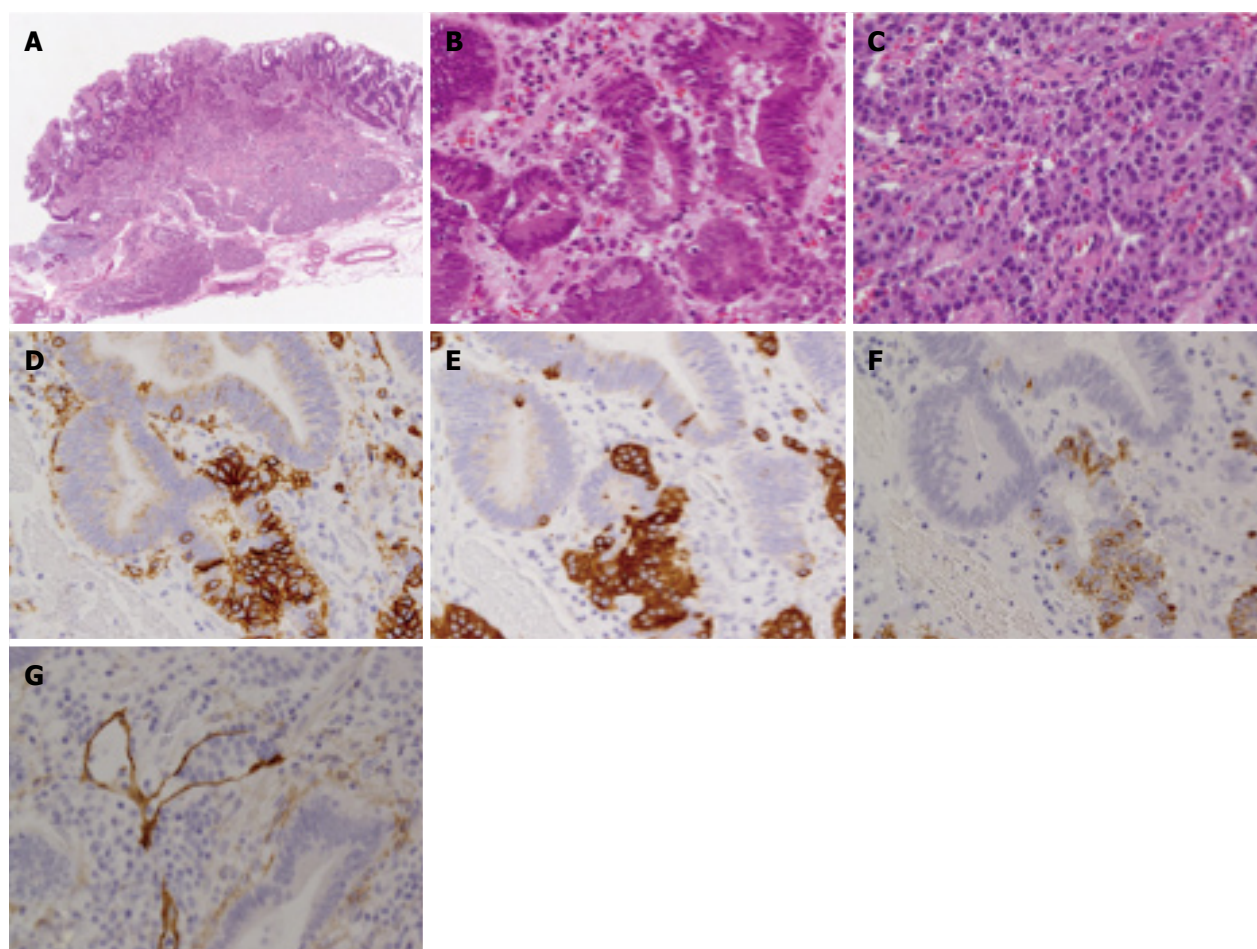


Figure 3 Histopathological results of the resected specimen. A: Low power view of a histological section demonstrates that the neuroendocrine tumor (NET) in the submucosa was covered with adenocarcinoma (HE stain $\times 40$); B: Both component cells had mixed in some parts and formed a tumor alveolar (H&E stain $\times 400$); C: High power view of NET in submucosa (HE stain $\times 400$); D: CD56; E: synaptophysin; F: Chromogranin A staining were positive in the NET cells; G: Lymphatic invasion of the NET component seen with D2-40 staining ($\times 400$).

adenocarcinoma with neuroendocrine features in a 67-year-old woman with acromegaly and thyroid papillary adenocarcinoma. Wen *et al.*^[7] reported a case of duodenal bulb adenocarcinoma with neuroendocrine features in a 63-year-old woman, which was treated by endoscopic mucosal resection. They stated that duodenal adenocarcinoma with neuroendocrine features is extremely rare^[7], and as per our review of the published literature, our report is likely the first instance of the gastric adenocarcinoma with duodenal NET resected by endoscopic treatment.

In this report, after ESD, we detected a tiny mass composed of nests of small uniform tumor cells that were typical of NET. The possible reasons the diagnosis of NET was overlooked before ESD are as follows: first, the whole tumor was located over the pylorus and its relatively large size made it difficult to observe the whole tumor precisely; and second, the NET component surface was covered with adenocarcinoma, which was also a protruded lesion.

Collision tumors are thought to arise from morphologically different neighboring neoplasms that do not intermingle^[8], but usually, it is not easy to

morphologically distinguish a collision-type from a composite-type tumor^[9]. It was difficult to determine whether the tumor was collision or composite-type in our patient as well: the immunohistochemical staining showed that the two lesions coexisted, suggesting a composite-type tumor, but the NET clinically presented in the duodenal part.

It was also difficult to distinguish histologically whether the adenocarcinoma derived from the stomach or duodenum as the ESD specimen did not include the muscle layer and it was impossible to detect the pyloric sphincter as a surgical specimen. Most of the tumor was present in the stomach and remainder gastric mucosa was atrophic, allowing the adenocarcinoma derived from the gastric epithelium to be diagnosed as not from duodenum. The NET was present in the end of the duodenal side of the specimen, and it was evident that the NET existed intraduodenally from the comparison marking dots of endoscopic findings and the analyte. We therefore judged clinically that the NET was from the duodenal epithelium (Figure 2).

Surgical treatment may have been one treatment

options, but as the tumor was diagnosed as early gastric cancer which had adaptation for ESD, surgery could be excessively invasive to the patient.

By the popularization of ESD, there have been some reports on cancer accompanied by submucosal tumor or NET^[5,8,9]. However, the coexistence of gastric NET and adenocarcinoma are rare, because gastric neuroendocrine tumors represent less than 1% of all gastric neoplasms^[8,10]. There have been no previous reports on endoscopic resection of gastric adenocarcinoma with duodenal NET; hence, we considered this case to be extremely rare.

We report the first case of early gastric cancer accompanied by duodenal NET, which was resected by ESD.

ACKNOWLEDGMENTS

We thank all medical staff and technicians of Yokohama City University Graduate School of Medicine.

COMMENTS

Case characteristics

A 78-year-old woman with medical history of hypertension and dyslipidemia presented with a protruded gastric tumor through the pyloric ring from the pyloric antrum.

Clinical diagnosis

Magnifying endoscopy with narrow-band imaging showed irregularities in the structures and vessels of the tumor, which the biopsy specimen led to the diagnosis of early gastric cancer.

Differential diagnosis

Gastric cancer with submucosal tumor invasion or duodenum cancer.

Laboratory diagnosis

All labs were within normal limits.

Imaging diagnosis

Computed tomography showed no remarkable metastasis.

Pathological diagnosis

A well differentiated adenocarcinoma composed of nests of small uniform tumor cells, which is typical of neuroendocrine tumor (NET).

Treatment

Endoscopic submucosal dissection.

Related reports

Although there were some reports about gastric collision tumor composed of epithelial and nonepithelial malignant neoplasm, malignant lymphoma was the most frequent finding and cases of gastric collision tumor composed of adenocarcinoma, and NET were rare. Duodenal adenocarcinoma with neuroendocrine features is also extremely rare and there were no previous report on endoscopic resection of gastric adenocarcinoma with duodenal NET.

Term explanation

NET is derived from enterochromaffin cells throughout the gastrointestinal (GI), pancreas and bronchopulmonary systems. The most common sites for primary GI carcinoid tumors in Japan are the rectum, stomach, and duodenum.

Experiences and lessons

Although we experienced an extremely rare case coincidentally, especially in the case of protruded tumor, the possibilities of collision tumor should be bear in mind.

Peer-review

In this paper the authors describe a duodenal neuroendocrine tumor resected by endoscopic submucosal dissection. This is a rare condition and manuscript is well written.

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