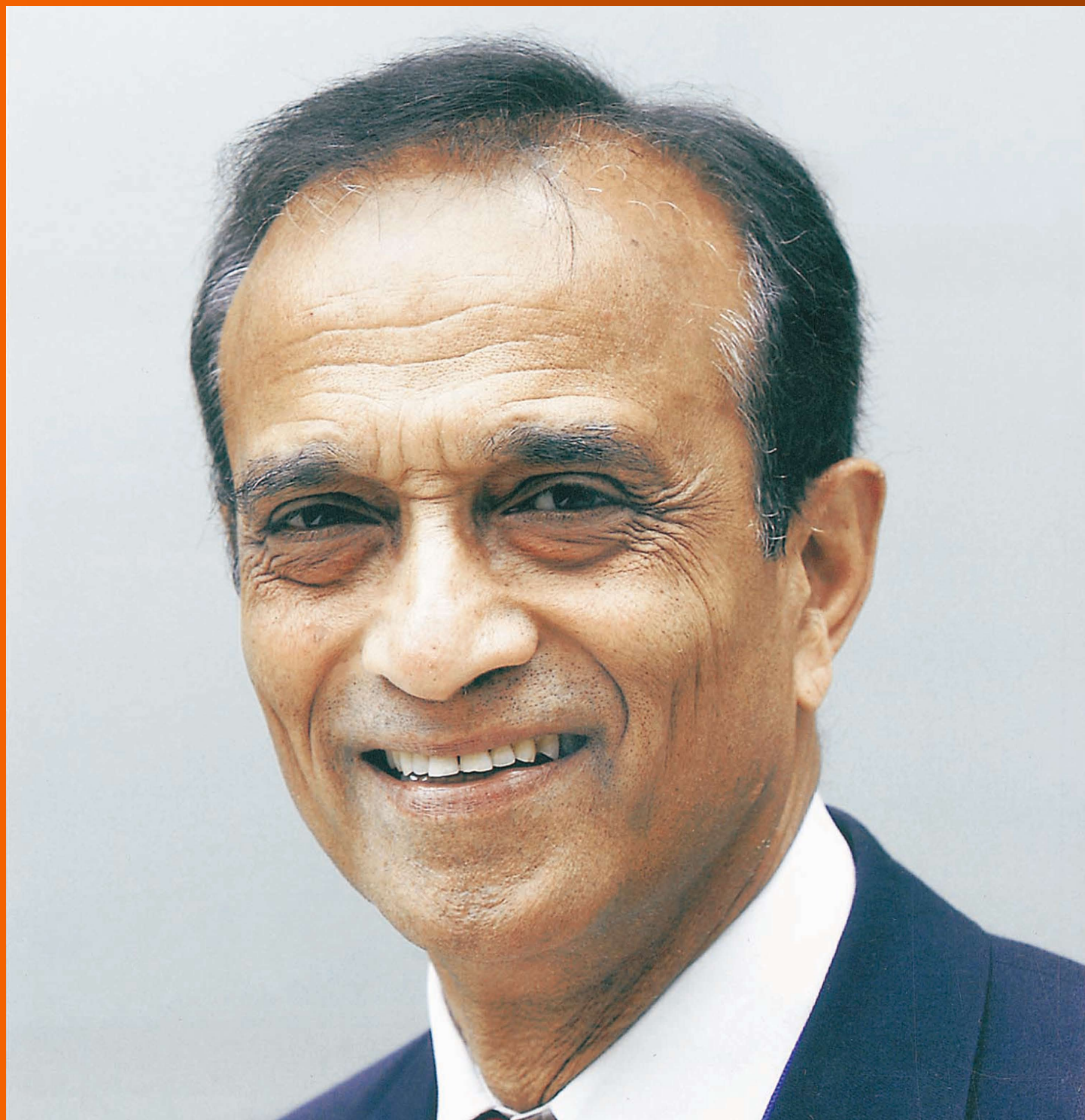


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Upfront surgery of small intestinal neuroendocrine tumors. Time to reconsider?

Kosmas Daskalakis, Apostolos V Tsolakis

Kosmas Daskalakis, Department of Surgical Sciences, Uppsala University, Uppsala 75185, Sweden

Apostolos V Tsolakis, Department of Oncology and Pathology, Karolinska Institute, Stockholm SE-171 76, Sweden

Apostolos V Tsolakis, Cancer Center Karolinska, Karolinska University Hospital Solna R8:04, Stockholm SE-171 76, Sweden

Apostolos V Tsolakis, Department of Gastrointestinal Endoscopy, Karolinska University Hospital Huddinge, Stockholm SE-141 86, Sweden

ORCID number: Kosmas Daskalakis (0000-0003-4224-8912); Apostolos V Tsolakis (0000-0002-6784-5572).

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Correspondence to: Apostolos V Tsolakis, MD, PhD, Doctor, Staff Physician, Department of Gastrointestinal Endoscopy, Karolinska University Hospital Huddinge, Stockholm SE-141 86, Sweden. apostolos.tsolakis@ki.se
Telephone: +46-8-58580000

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Abstract

Small intestinal neuroendocrine tumors (SI-NETs) may demonstrate a widely variable clinical behavior but usually it is indolent. In cases with localized disease, locoregional resective surgery (LRS) is generally indicated with a curative intent. LRS of SI-NETs is also the recommended treatment when symptoms are present, regardless of the disease stage. Concerning asymptomatic patients with distant metastases, prophylactic LRS has been traditionally suggested to avoid possible future complications. Even the current European Neuroendocrine Tumor Society guidelines emphasize a possible effect of LRS in Stage IV SI-NETs with unresectable liver metastases. On the contrary, the 2017 National Comprehensive Cancer Network Guidelines on carcinoid tumors do not support the resection of a small, asymptomatic, relatively stable primary tumor in the presence of unresectable metastatic disease. Furthermore, a recent study revealed no survival advantage for asymptomatic patients with distant-stage disease who underwent upfront LRS. At the aforementioned paper, it was suggested that delayed surgery as needed was comparable with the upfront surgical approach in terms of postoperative morbidity and mortality, the length of the hospital stay and the rate of incisional hernia repairs but was associated with fewer reoperations for bowel obstruction. On the other hand, it is also important to note that some patients might benefit from a prophylactic surgical approach and our attention should focus on identifying this patient population.

Key words: Small intestinal neuroendocrine tumors; Locoregional resective surgery

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Core tip: Upfront locoregional resective surgery of small intestinal neuroendocrine tumors is the mainstay treatment when radical resection is feasible or when symptoms are present, regardless of the disease stage. However, in the light of contemporary evidence, the traditional upfront surgical approach is challenged regarding patients with distant metastases without local tumor-related symptoms.

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INTRODUCTION

Small intestinal neuroendocrine tumors (SI-NETs) have an indolent clinical course and are often diagnosed at a late stage^[1]. In patients without distant metastases, locoregional resective surgery (LRS) is generally indicated with a curative intent. However, in patients with distant-stage disease, LRS is generally not considered curative, although sometimes liver surgery or local ablative treatments are undertaken after or before radical LRS.

Even in the era of a broad panel of novel, targeted and systemic therapies for SI-NETs, recurrence after perceived radical liver resection is still very common, and neither liver resection nor radiofrequency ablation of liver metastases has unequivocally been found to prolong survival^[2,3]. Therefore, even with the intention to achieve macroscopic radicality and cure, liver procedures for SI-NETs should generally be considered palliative^[4].

STUDY ANALYSIS

Many patients with distant-stage disease may present with distinct clinical symptoms and signs due to hormonal excess and/or with local tumor-related symptoms causing abdominal pain, obstruction and/or an impaired blood supply to the intestines. These patients with local tumor-related symptoms generally undergo LRS at the time of diagnosis. Some patients may undergo an acute laparotomy because of an intestinal obstruction of unknown etiology. Others will undergo palliative surgery for a partial intestinal obstruction, bleeding, ischemic complications due to a tumor mass, or even for symptom relief in the cases of hormonal syndrome refractory to medical therapy.

The extension of mesenteric lymph node metastases below or above the horizontal part of the duodenum is a crucial factor for treatment since a number of patients will display mesenteric lymph node metastases in the root of the mesentery, with associated fibrosis, encasing

the superior mesenteric vessels. These tumors are then usually considered inoperable. Palliative, minimally invasive measures such as stenting of the superior mesenteric vein have been applied to symptomatic patients with bulky mesenteric disease since LRS in these patients may be complicated and endanger circulation to substantial parts of the bowel^[5].

Generally, for tumors originating in the proximal ileum and jejunum, segmental small intestinal resection is performed. However, for primary tumors located near the ileocecal valve in the distal ileum, ileocecal resection or right hemicolectomy is performed, with the latter possibly combined with improved clearance of regional lymph node metastases. Even though the latest Surveillance Epidemiology and End Results report challenges the prognostic significance of lymphatic metastasis for SI-NETs with locoregional disease only, there are certain biases and limitations in these data^[6].

In asymptomatic patients with distant metastases, prophylactic LRS has been traditionally advocated to avoid a future intestinal obstruction, ischemia, perforation or bleeding. The survival rates of these patients after LRS, as reported in retrospective cohort studies, are probably largely influenced by both the selection bias and immortal time bias. Generally, there are differences in contemporary literature from up-to-date guidelines about approaching endocrine disorders^[7]. The current ENETS guidelines emphasize a possible effect of LRS in Stage IV SI-NETs with unresectable liver metastases, but these guidelines are based on the information gathered from the abovementioned cohort studies^[8,9].

On the other hand, the 2017 National Comprehensive Cancer Network Guidelines on carcinoid tumors advocate against resection of a small, asymptomatic, relatively stable primary tumor in the presence of unresectable metastatic disease^[10]. A recent study has revealed no survival advantage for asymptomatic patients with distant-stage disease who underwent upfront LRS^[11]. Interestingly, delayed surgery as needed was comparable with the upfront surgical approach in terms of postoperative morbidity and mortality, the length of the hospital stay and the rate of incisional hernia repairs but was associated with fewer reoperations for bowel obstruction^[12]. These results are also consistent with the Rotterdam group findings that confirmed that there is no benefit of prophylactic surgery for overall survival^[12]. However, it is also important to note that while patients with disseminated SI-NETs may not benefit from upfront prophylactic surgery, some patient populations might, *e.g.*, older patients or those with large tumors and patients with progressive locoregional disease^[13]. Importantly, to be able to identify patients who might benefit from a prophylactic surgical approach, more insight is needed into the development of mesenteric fibrosis in SI-NETs^[9].

PERSPECTIVE

In conclusion, LRS retains its value in the treatment of patients with SI-NETs when radical resection is feasible

or symptomatic disease is present, regardless of the disease stage.

However, current evidence challenges the traditional view that extensive LRS needs to be performed in patients with distant metastases in the absence of local tumor-related symptoms. A more conservative approach, with delayed LRS as clinically indicated, may be reasonable for the subset of asymptomatic SI-NET patients with distant-stage disease. This revised approach may complete the armamentarium of systemic and liver-directed treatments as indicated per patient.

REFERENCES

- 1 **Norlén O**, Stålberg P, Öberg K, Eriksson J, Hedberg J, Hessman O, Janson ET, Hellman P, Åkerström G. Long-term results of surgery for small intestinal neuroendocrine tumors at a tertiary referral center. *World J Surg* 2012; **36**: 1419-1431 [PMID: 21984144 DOI: 10.1007/s00268-011-1296-z]
- 2 **Elias D**, Lefevre JH, Duvillard P, Goéré D, Dromain C, Dumont F, Baudin E. Hepatic metastases from neuroendocrine tumors with a "thin slice" pathological examination: they are many more than you think. *Ann Surg* 2010; **251**: 307-310 [PMID: 20010089 DOI: 10.1097/SLA.0b013e3181bdf8cf]
- 3 **Sarmiento JM**, Heywood G, Rubin J, Ilstrup DM, Nagorney DM, Que FG. Surgical treatment of neuroendocrine metastases to the liver: a plea for resection to increase survival. *J Am Coll Surg* 2003; **197**: 29-37 [PMID: 12831921 DOI: 10.1016/S1072-7515(03)00230-8]
- 4 **Norlén O**, Stålberg P, Zedenius J, Hellman P. Outcome after resection and radiofrequency ablation of liver metastases from small intestinal neuroendocrine tumours. *Br J Surg* 2013; **100**: 1505-1514 [PMID: 24037573 DOI: 10.1002/bjs.9262]
- 5 **Daskalakis K**, Karakatsanis A, Stålberg P, Norlén O, Hellman P. Clinical signs of fibrosis in small intestinal neuroendocrine tumours. *Br J Surg* 2017; **104**: 69-75 [PMID: 27861745 DOI: 10.1002/bjs.10333]
- 6 **Chen L**, Song Y, Zhang Y, Chen M, Chen J. Exploration of the Exact Prognostic Significance of Lymphatic Metastasis in Jejunoileal Neuroendocrine Tumors. *Ann Surg Oncol* 2018; **25**: 2067-2074 [PMID: 29748891 DOI: 10.1245/s10434-018-6511-9]
- 7 **Isik A**, Firat D, Yilmaz I, Peker K, Idiz O, Yilmaz B, Demiryilmaz I, Celebi F. A survey of current approaches to thyroid nodules and thyroid operations. *Int J Surg* 2018; **54**: 100-104 [PMID: 29709542 DOI: 10.1016/j.ijsu.2018.04.037]
- 8 **Capurso G**, Rinzivillo M, Bettini R, Boninsegna L, Delle Fave G, Falconi M. Systematic review of resection of primary midgut carcinoid tumour in patients with unresectable liver metastases. *Br J Surg* 2012; **99**: 1480-1486 [PMID: 22972490 DOI: 10.1002/bjs.8842]
- 9 **Niederle B**, Pape UF, Costa F, Gross D, Kelestimur F, Knigge U, Öberg K, Pavel M, Perren A, Toumpanakis C, O'Connor J, O'Toole D, Krenning E, Reed N, Kianmanesh R; Vienna Consensus Conference participants. ENETS Consensus Guidelines Update for Neuroendocrine Neoplasms of the Jejunum and Ileum. *Neuroendocrinology* 2016; **103**: 125-138 [PMID: 26758972 DOI: 10.1159/000443170]
- 10 **National Comprehensive Cancer Network**. NCCN guidelines version 3.2017. Neuroendocrine tumors of the gastrointestinal tract, lung and thymus (carcinoid tumors). Available from: URL: https://www.nccn.org/professionals/physician_gls/pdf/neuroendocrine.pdf
- 11 **Daskalakis K**, Karakatsanis A, Hessman O, Stuart HC, Welin S, Tiensuu Janson E, Öberg K, Hellman P, Norlén O, Stålberg P. Association of a Prophylactic Surgical Approach to Stage IV Small Intestinal Neuroendocrine Tumors With Survival. *JAMA Oncol* 2018; **4**: 183-189 [PMID: 29049611 DOI: 10.1001/jamaoncol.2017.3326]
- 12 **Blažević A**, Zandee WT, Franssen GJH, Hofland J, van Velthuisen MF, Hofland LJ, Feelders RA, de Herder WW. Mesenteric fibrosis and palliative surgery in small intestinal neuroendocrine tumours. *Endocr Relat Cancer* 2018; **25**: 245-254 [PMID: 29255095 DOI: 10.1530/ERC-17-0282]
- 13 **Wu L**, Fu J, Wan L, Pan J, Lai S, Zhong J, Chung DC, Wang L. Survival outcomes and surgical intervention of small intestinal neuroendocrine tumors: a population based retrospective study. *Oncotarget* 2017; **8**: 4935-4947 [PMID: 27903960 DOI: 10.18632/oncotarget.13632]

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Helicobacter pylori and extragastric diseases: A review

Antonietta Gerarda Gravina, Rocco Maurizio Zagari, Cristiana De Musis, Lorenzo Romano, Carmelina Loguercio, Marco Romano

Antonietta Gerarda Gravina, Cristiana De Musis, Lorenzo Romano, Carmelina Loguercio, Marco Romano, Dipartimento di “Medicina di Precisione”, UOC Epatogastroenterologia, Università della Campania “Luigi Vanvitelli”, Napoli 80131, Italy

Rocco Maurizio Zagari, Dipartimento Di Scienze Mediche e Chirurgiche, Università di Bologna, Bologna 40138, Italy

ORCID number: Antonietta Gerarda Gravina (0000-0001-8049-0115); Rocco Maurizio Zagari (0000-0001-9949-8619); Cristiana De Musis (0000-0001-7011-5047); Lorenzo Romano (0000-0002-6581-7930); Carmelina Loguercio (0000-0002-6863-6510); Marco Romano (0000-0002-3271-349X).

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Correspondence to: Marco Romano, MD, PhD, Full Professor, Dipartimento di “Medicina di Precisione”, UOC Epatogastroenterologia, Università degli Studi della Campania “Luigi Vanvitelli”, Via Pansini, 5, Napoli 80131, Italy. marco.romano@unicampania.it
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Abstract

Helicobacter pylori (*H. pylori*) infection is very common and affects approximately half of the world population. It causes gastric diseases, but some authors have reported an association of *H. pylori* infection with other systemic manifestations beginning in 1994. The list of potential effects of *H. pylori* outside the stomach includes a number of extragastric manifestations and we focused on neurological, dermatological, hematologic, ocular, cardiovascular, metabolic, allergic, and hepatobiliary diseases. This review discusses these important reported manifestations that are not related to the gastrointestinal tract.

Key words: *Helicobacter pylori*; Extragastric disease; Neurological diseases; Dermatological diseases; Hematologic diseases; Ocular diseases; Cardiovascular diseases; Metabolic diseases; Allergic diseases; Hepatobiliary diseases

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Core tip: *Helicobacter pylori* (*H. pylori*) infection is a common infection that can cause gastric and extragastric diseases. A considerable amount of evidence links *H. pylori* infection with extragastric diseases, and in many of these diseases there is a clear beneficial effect of eradication therapy. This review summarizes the *H. pylori*-related extragastric manifestations of major interest that have been reported in the scientific literature, such as neurological, dermatological, hematologic, ocular, cardiovascular, metabolic, allergic

and hepatobiliary disease manifestations.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic, spiral-shaped and flagellated bacterium infecting about half the world's population whose main reservoir is the human stomach. The prevalence of infection varies by geographic area, age, ethnicity and socioeconomic status; in fact, the prevalence is higher in developing countries and in those with poor socio-economic conditions^[1-4]. *H. pylori* possesses microbiological characteristics that allow it to survive in extremely adverse conditions such as the gastric acidic environment. Transmission of the infection occurs mainly through the oral-fecal route^[5], in particular through contaminated water and food. Oral-oral transmission is also possible, as shown by the isolation of the bacterium in saliva and dental plaque^[6].

H. pylori infection is the main cause of chronic gastritis and peptic ulcer disease^[7]. *H. pylori* has a determinant pathogenic role in the development of distal gastric adenocarcinoma and gastric mucosa associated lymphoid tissue (MALT) lymphoma; in fact, it can contribute to gastric carcinogenesis by stimulating gastric cell proliferation without counterbalancing with adequate apoptosis^[8,9]. The spectrum of gastroduodenal diseases associated with the evolution of *H. pylori* infection is wide. A large proportion of infected subjects (approximately 80%-85%) develop mild antrum and body gastritis associated with an alteration of gastric homeostasis characterized by hypergastrinemia with normal levels of gastric acid secretion. These subjects are unlikely to develop severe clinical conditions. Approximately 10%-15% of infected individuals develop prevalent antrum gastritis, in which hypergastrinemia is associated with increased gastric secretion with the possible development of duodenal ulceration^[10]. A lower percentage (approximately 1%-2%) develop a prevalent body gastritis in response to the infection that is associated with multifocal atrophic gastritis, increased gastrinemia, hypo-chlorhydria and the possible development of gastric adenocarcinoma. The reason why *H. pylori* infection is associated with different gastric phenotypes with different clinical outcomes is not fully known, but it is likely that the interaction among bacterial virulence factors (e.g., CagA, urease, VacA, and babA2), environmental factors (e.g., socioeconomic conditions, nutrition, and exposure to toxic substances) and genetic

substrates of the host [e.g., IL1B gene cluster and tumor necrosis factor- α (*TNF α*) gene polymorphism] play an important role^[11]. The first reports of the association between *H. pylori* infection and extra-gastric diseases were by Mendall *et al.*^[12] in 1994. There are several clinical extra-gastric manifestations associated with *H. pylori* infection that have been reported to date, making this a very interesting and debated topic. We summarize the data in the literature about extra-digestive diseases associated with *H. pylori* infection and focus our attention on the following conditions that are potentially linked to *H. pylori* infection: neurological, dermatological, hematologic, ocular, cardiovascular, metabolic and allergic diseases. For some of these diseases, only one association is described without a clear explanation of the pathogenic mechanism; however, for others, as we will see later, the association is so strong and the pathogenic mechanism is so clear that guidelines for the treatment of *H. pylori* infection suggest that in these conditions, *H. pylori* infection should be determined and, if present, should be treated with eradication therapy^[13].

NEUROLOGICAL DISEASES

Several neurological disorders are associated with *H. pylori* infection. There are studies in the literature that report a positive predictive value between *H. pylori* infection and stroke; however, in 2013, in a prospective cohort analysis performed on 9885 subjects with stroke, Chen *et al.*^[14] not only reported no increased mortality but actually found that people infected with *H. pylori* had a lower mortality than the general population. In a recent metanalysis, Wang *et al.*^[15] demonstrated that chronic *H. pylori* infection and the presence of CagA-positive strains were statistically significant risk factors for ischemic stroke. The underlying pathogenic mechanism is not yet known, but it has been hypothesized that *H. pylori* increases the expression of a number of mediators of inflammation and activates platelets and factors involved in coagulation^[16]. Difference in the study population and in the methods to assess *H. pylori* infection might partially explain the discrepancy between different studies.

Another neurologic disease that has been linked to *H. pylori* infection is Alzheimer's disease (AD). There are several studies concerning *H. pylori* and dementia. Huang *et al.*^[17] showed a 1.6 times greater risk of developing AD in *H. pylori*-infected people than in non-infected people, supporting a possible role of *H. pylori* in the pathophysiology of AD. Roubaud Baudron *et al.*^[18] also reported a 1.5 times greater risk of developing dementia over a 20-year follow-up period in infected people than in non-infected people. Beydoun *et al.*^[19] also reported an association between *H. pylori* infection and reduced cognitive ability. There are also several studies that report that eradication of infection can positively influence the manifestations of AD^[20-22]. In 2016, Kountouras *et al.*^[23] showed that in patients

with AD and *H. pylori* infection there was an increased prevalence of apolipoprotein E (ApoE) 4 polymorphism compared with non-infected patients. The ApoE 4 polymorphism is the strongest genetic risk factor for AD^[23,24]. In 2009, the same author reported significantly higher levels of anti-*H. pylori*-specific antibodies (anti-*H. pylori* IgG) in the cerebrospinal fluid (CSF) and serum from patients with AD than that in the CSF and serum from age-matched subjects with normal cognition. The same research group demonstrated a significant correlation between the severity of disease and levels of anti-*H. pylori* IgG in the CSF of these patients^[25]. One hypothesis to explain the association between *H. pylori* and AD is that *H. pylori* might access the brain *via* an oral-nasal-olfactory pathway thus leading to neurodegeneration. In fact, olfactory performance is reduced in 90% of patients with AD, and significant olfactory bulb atrophy, as evaluated by imaging techniques, is present in these patients^[26-29]. Another hypothesis is that *H. pylori* may access the brain *via* monocytes infected with *H. pylori* (due to *H. pylori* replication in autophagic vesicles) through a disrupted blood-brain barrier (BBB), which is also called the "Trojan horse theory"; this may lead to increased production of inflammatory mediators, such as TNF- α , which in turn may cause BBB disruption by up-regulation of metalloproteinases^[30]. A third hypothesis is that *H. pylori* may access to brain through a fast retrograde neural pathway from the gastrointestinal tract (GIT), leading to neurodegeneration^[30]. A study conducted in Japan^[31] did not confirm the association between *H. pylori* infection and AD. However the high prevalence of *H. pylori* in controls might partially explain the difference with studies conducted in Western countries where the prevalence of the infection is lower. Furthermore, Chang *et al.*^[32] in partial support of a role for *H. pylori* in AD, demonstrated that eradication of the infection led to a decreased progression of the neurological disease.

Multiple sclerosis (MS) is a chronic demyelinating disease that affects the central nervous system. Mohebi *et al.*^[33] showed an inverse relationship between *H. pylori* infection and MS; however, other authors have shown positivity of markers for optic neuromyelitis in *H. pylori*-positive patients. The administration of *H. pylori* SS1 antigen in an experimental model of MS [experimental autoimmune encephalomyelitis (EAE)], indicates the presence of immunomodulating properties of *H. pylori*, suggesting a possible effect of *H. pylori* infection in the pathophysiology of MS^[34]. Cook *et al.*^[35] showed a probable protective role of *H. pylori* against EAE by inhibiting both Th1 and Th17 responses. *H. pylori* infection seems to be a risk factor for the development of aquaporin 4 (AQP4) antibodies in MS, through molecular mimicry between AQP4 and bacterial AQP^[14]. Therefore, based on these studies, whether *H. pylori* infection may cause MS is still controversial.

Another disease of great neurological interest for which an association with *H. pylori* infection has been reported is Parkinson's disease (PD). PD is caused

by the degeneration of the dopaminergic neurons of the substantia nigra pars compacta of the basal ganglia system. A meta-analysis by Shen *et al.*^[36] in 2017, evaluating eight eligible studies involving 33125 participants, demonstrated that *H. pylori* infection might be associated with the risk of PD. A recent study by Huang *et al.*^[37] in the general population in Taiwan demonstrated that *H. pylori* infection was significantly associated with an increased risk of PD among individuals who were ≥ 60 years old but not among those < 60 years old. *H. pylori* infection may affect L-3,4-dihydroxyphenylalanine (L-dopa) bioavailability, which is used in the treatment of PD, by disrupting the duodenal mucosa, which is the site of absorption of L-dopa^[38]. The eradication of the infection appears to be associated with greater bioavailability of L-dopa and is associated with better clinical outcomes^[39,40]. Several studies have demonstrated that pro-inflammatory cytokines associated with chronic gastrointestinal disease can induce brain inflammation through a disruption of the BBB and death of dopaminergic neurons and may eventually be responsible for parkinsonism^[41-43]. The association between PD and *H. pylori* infection seems, therefore, to be strongly supported by literature reports.

Guillain-Barré syndrome (GBS) is an acute autoimmune neuropathy characterized by progressive paralysis of the limbs with a distal-proximal pattern (the legs being affected first and the arms later). It can cause life-threatening complications, particularly if there is respiratory muscle involvement or autonomic nervous system involvement. The disease is usually triggered by an infection. *Campylobacter jejuni* (*C. jejuni*), the most prevalent cause of bacterial gastroenteritis, has been associated with GBS and the possible pathogenic mechanism involves molecular mimicry between peripheral nerve gangliosides and *C. jejuni* lipopolysaccharides (LPSs). In fact sialic acid, a characteristic component of human gangliosides, is present among the surface antigens of *C. jejuni*. *H. pylori* has a high-molecular weight sequence in LPSs that is detected in one third of *C. jejuni* serotypes. Therefore a molecular mimicry between *H. pylori* antigens and peripheral nerve gangliosides might be responsible for the association between *H. pylori* infection and GBS^[16,44,45]. Some authors demonstrated that the presence of serum anti-*H. pylori* IgG in patients with GBS was significantly higher than that in controls. They also demonstrated that the CSF was positive for anti-*H. pylori* IgG in 80% of patients and in only 20% of controls^[16]. Chiba *et al.*^[46] demonstrated IgG antibodies to vacuolating cytotoxin A (VacA) of *H. pylori* in the CSF of patients with GBS. In this study, the authors found sequence homology between VacA and the human ATPase A subunit, suggesting that antibodies to VacA might bind to ion channels in Schwann cells, resulting in the demyelination of motor neurons in these patients. A limitation of all these studies is the small sample size and, for this reason, studies with a greater sample size are needed to demonstrate a real cause-

effect relationship between *H. pylori* and GBS.

DERMATOLOGICAL DISEASES

Rosacea is the most common dermatological disease associated with *H. pylori* infection. It is a chronic facial dermatitis that manifests as erythema and cutaneous lesions characterized by much dilated red superficial capillaries, called telangiectasia, and its etiology remains unknown. Although the etiopathogenesis is not fully known, an element commonly found in patients with rosacea is the presence of gastrointestinal disorders. Several studies have suggested a potential relationship to *H. pylori* infection; however, the correlation between *H. pylori* infection and rosacea is still debated. Our group has demonstrated *H. pylori* infection in 48.9% of patients with rosacea, while only 26.7% of patients in the control group were infected with *H. pylori*. We demonstrated an improvement with partial or total regression of rosacea skin lesions after successful *H. pylori* eradication in 96.9% of patients^[47]. Argenziano *et al*^[48] reported *H. pylori* infection in 81% of patients with rosacea, and almost all of the patients housed CagA-positive strains. El-Khalawany *et al*^[49] demonstrated that papular rosacea responded better to eradication therapy than erythematous rosacea did. Based on the majority of studies one may therefore suggest to test for and eradicate *H. pylori* infection in patients with rosacea.

Psoriasis is an autoimmune chronic inflammatory disease of the skin that is not infectious or contagious and is usually of a chronic and recurring nature. The association between *H. pylori* infection and psoriasis is still controversial. In fact some authors demonstrated neither a significant relationship between psoriasis and *H. pylori*^[50,51], nor a significant relationship between psoriasis severity and the serum levels of IgG anti-*H. pylori*^[51]. However, other authors demonstrated that *H. pylori* infection is more common in patients with psoriasis than in healthy controls^[52,53] and that prevalence of *H. pylori* infection was higher in patients with severe psoriasis (79%) compared to those with moderate (69.5%) or mild (46.2%) disease^[53,54]. Finally, in support of a causative role for *H. pylori* in psoriasis, an interventional study by Onsun *et al*^[55] demonstrated that eradication of *H. pylori* infection caused more rapid improvement than treatment with acitretin alone.

Chronic urticaria (CU) is characterized by the appearance of a more or less itchy rash, and its unique lesion is the wheal. Some research groups have reported a higher prevalence of *H. pylori* infection in patients with CU^[56,57]. Campanati *et al*^[58] demonstrated no difference in the presence of *H. pylori* infection between patients with CU and apparently healthy people, but they reported a significant improvement in skin lesions after eradication therapy. Yoshimasu *et al*^[59] demonstrated that patients with CU infected with *H. pylori* had a cure rate of approximately 56% and that

none of the patients with *H. pylori* infection were cured if they were treated with antihistamine therapy alone.

Data regarding the association between alopecia aerata (AA) and *H. pylori* infection are discordant. In fact, some authors have reported a higher prevalence of infection in patients with AA, but other authors did not confirm this association^[60,61]. Differences in methodology might account for the discrepancy of results related to the role of *H. pylori* in CU or AA and, therefore, larger population studies and controlled trials to obtain more accurate evidence about these associations are needed.

Autoimmune bullous diseases (AIBD) are a group of dermatological diseases including pemphigus, pemphigoid, epidermolysis bullosa acquisita, dermatitis herpetiformis, and linear immunoglobulin A disease^[62,63]. Very few published studies have investigated the association between AIBD and *H. pylori* infection. Sagi *et al*^[64] and Mortazavi *et al*^[65] demonstrated that anti-*H. pylori* IgG was higher in patients with AIBD than in controls. In the Mortazavi *et al*^[65] study, the prevalence of *H. pylori* infection was as high as 79.3% in the AIBD group.

Schöenlein-Henoch purpura (SHP) is an immune condition characterized by the deposition of immunoglobulin A in the skin and in other organs, such as the kidneys, joints, and gastrointestinal tract. The onset of this disease is characterized by the appearance of purple skin lesions. There are a few reports that support the association between *H. pylori* infection and SHP, demonstrating an improvement in skin lesions following the successful eradication of the infection^[66-68]. Clinical studies with a great sample size are necessary prior to draw a firm conclusion regarding the role of *H. pylori* in SHP.

HEMATOLOGIC DISEASES

Iron deficiency anemia (IDA) is well known to be associated with *H. pylori* infection. In 1991, Blecker *et al*^[69] described a case of hemorrhagic gastritis linked to *H. pylori* infection and showed a possible relationship between *H. pylori* infection and IDA. Subsequently, 5 meta-analyses concluded that there was a close association between infection and IDA^[70-74]. Guidelines for the treatment of *H. pylori* infection indicate that the infection should be eradicated in cases of IDA^[13]. The reason why IDA is not always associated with *H. pylori* infection is not known. Azab *et al*^[75] studied the role of hepcidin, which is a small protein responsible for regulating iron recycling and the balance of iron in the body. Hepcidin, which is produced in the liver, regulates the absorption of iron from enterocytes and its release from macrophages^[76]. *H. pylori* infection upregulates hepcidin serum levels, thus attenuating the response to iron therapy^[77]. Senkovich *et al*^[78] demonstrated that *H. pylori* is able to acquire iron from host transferrin and lactoferrin. Yokota *et al*^[79] demonstrated that some polymorphisms within the *H. pylori* neutrophil-

activating protein were more frequent in strains from IDA patients than in those from non-IDA patients. *H. pylori* that harbors these polymorphisms internalizes iron more rapidly than other strains^[79]. Inorganic iron dissolves best in a highly acidic environment, and *H. pylori* infection may reduce the bioavailability of dietary iron. Patients infected with CagA-positive strains and those with refractory IDA have high serum levels of TNF α , which is a pro-inflammatory cytokine that can induce anemia^[80,81]. Hershko *et al.*^[82] demonstrated that 64%-75% of patients with IDA and *H. pylori* infections had a complete disappearance of IDA after *H. pylori* eradication. Other possible causes of IDA in patients with *H. pylori* infections are chronic or acute bleeding due to erosive gastritis or concomitant therapy with non-steroidal anti-inflammatory drugs including aspirin^[83-86].

Vitamin B₁₂ is the coenzyme of many important enzymatic reactions in the human body that lead to DNA synthesis^[87]. Lahner *et al.*^[88] showed an association between low serum levels of vitamin B₁₂ and *H. pylori* infection in a systematic review evaluating 17 studies involving 2454 patients. Homocysteine is a component of the vitamin B₁₂ metabolic pathway^[89], and some authors have demonstrated a relationship between low serum levels of vitamin B₁₂ and increases in serum homocysteine associated with *H. pylori* infection^[90]. Increased serum levels of vitamin B₁₂ and decreased serum levels of homocysteine have been reported after *H. pylori* eradication^[87]. The pathophysiological mechanism of the association between vitamin B₁₂ deficiency and *H. pylori* infection is also unknown. The absorption of vitamin B₁₂ may be compromised in corpus-predominant *H. pylori*-related gastritis. Chronic vitamin B₁₂ deficiency can cause a pernicious anemia and peripheral neuropathy and lesions of the spinal cord^[91].

Primary immune thrombocytopenia (ITP), which was previously called idiopathic thrombocytopenic purpura and autoimmune thrombocytopenic purpura, is an autoimmune disorder characterized by an isolated thrombocytopenia (a peripheral blood platelet count of $100 \times 10^9/L$) in the absence of other causes^[87]. The first case of association between *H. pylori* infection and ITP was described in Spain in 1999^[92]. In the world literature, there are many authors who have reported this association, and a significant increase in platelet count following the eradication of *H. pylori* infection varies from 32% to 100% in Italian studies^[93,94] and from 26% to 100% in the overall literature^[95,96]. There are only a few published studies that do not describe a positive association between *H. pylori* infection and ITP, but this may be explained by the low prevalence of the infection in these countries^[97-100]. *H. pylori*-infected patients who have a particular polymorphism in IL- β , the IL- β (-511) T allele, are more likely to develop ITP than uninfected ITP patients^[80,101]. The pathogenesis of ITP associated with *H. pylori* infection is most likely multifactorial. Various mechanisms involved in this autoimmune process in patients with *H. pylori* infection

have been described, and one mechanism does not exclude the other. One of these mechanisms is the modulation of the Fc γ receptor balance, which is related to the activation of monocytes/macrophages and the relationship to the inhibitory receptor Fc γ RIIB. Monocytes from *H. pylori*-infected patients exhibited an enhanced phagocytic capacity and low levels of inhibitory Fc γ RIIB, leading to increased monocyte function with autoreactivity with B and T lymphocytes. This may cause the production of autoantibodies by lymphocyte B against circulating platelets. Eradication of *H. pylori* in ITP causes an upregulation of Fc γ RIIB expression in monocytes, increased platelet recovery and suppression of antigen presentation by macrophages with the subsequent inhibition of T- and B-cell responses to platelet antigens^[87,102]. Another mechanism potentially involved in *H. pylori*-associated ITP is molecular mimicry between platelet surface glycoproteins, including glycoprotein IIIa, and amino acid sequences of virulence factors, such as VacA, CagA and urease B^[87,103]. Anti-*H. pylori* IgG can induce opsonization of platelets by binding to *H. pylori*, von Willebrand's factor, and GPIb^[102,104].

The putative mechanisms responsible for the association between *H. pylori* infection, IDA, Vitamin B₁₂ deficiency and ITP are summarized in Figure 1.

Other unrecognized hematologic disorders that are associated with *H. pylori* infection are autoimmune neutropenia (AN), anti-phospholipid syndrome (AS), and plasma cell dyscrasias (PCD). The association between AN and *H. pylori* infection was described for the first time by Cicconi *et al.*^[105] in 2001 and was subsequently described by other authors^[105,106]. AN (defined as 400 neutrophils per μL) may dramatically improve after *H. pylori* eradication^[87,107,108]. The association between *H. pylori* infection and PCD, including monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma, solitary plasmacytomas, plasma cell leukemia, Waldenstrom macroglobulinemia and other chronic myeloproliferative diseases of B lymphocytes, is supported by some authors but not by others^[107,109-111]. This relationship maybe a result of chronic antigenic stimulation of B lymphocytes by *H. pylori*^[84].

OCULAR DISEASES

Ocular diseases described in association with *H. pylori* infection are open-angle glaucoma (OAG), central serous chorioretinitis (CSC) and blepharitis (B). There are studies that show a prevalence of *H. pylori* infection that is approximately two-fold higher in patients with OAG than in controls. However, other authors including Galloway *et al.*^[112] and Kurtz *et al.*^[113] did not find a higher prevalence of infection in patients with open-angle glaucoma. Zeng *et al.*^[114] published a meta-analysis about this association, evaluating ten studies, and suggested a statistically significant association between *H. pylori* infection and OAG. In further support of an association between *H. pylori* infection and

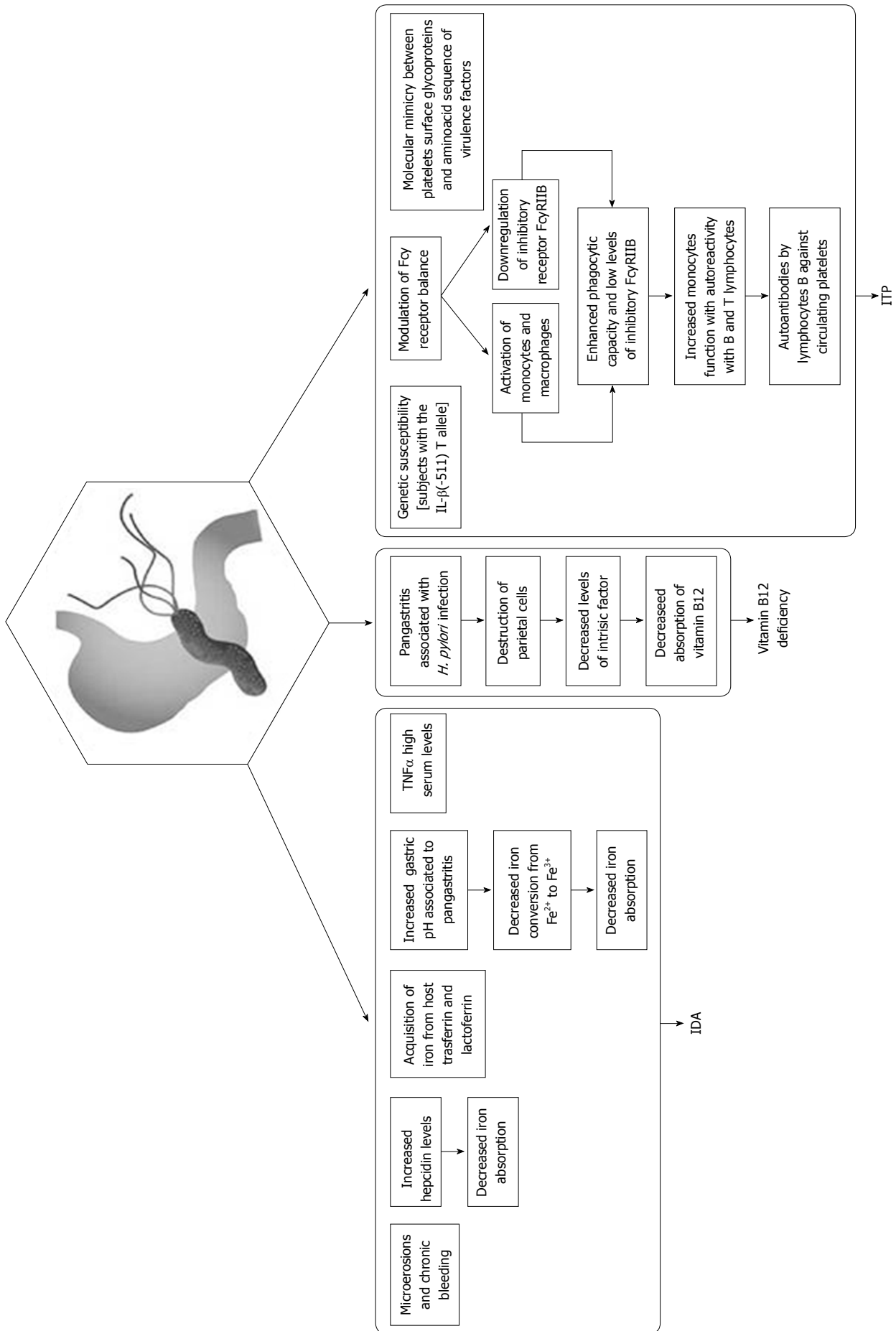


Figure 1 Hematologic diseases associated to *Helicobacter pylori* infection.

OAG, successful eradication of the infection has been reported to lead to an improvement in ocular pressure parameters in treated patients compared with the patients who do not have the infection eradicated^[80].

CSC is an ocular disease that causes a temporary reduction in central vision and usually affects only one eye. In the different phases of activity, it is characterized by the presence of liquid passing under the retina, which tends to accumulate under the macula. This results in blurred or distorted vision, with a reduction in visual acuity that can persist even after reabsorption of the fluid if it does not take place rapidly^[115]. In a systematic review and meta-analysis about the risk factors for CSC published in 2016, Liu *et al.*^[116] concluded that *H. pylori* infection was a possible risk factor for the occurrence of CSC. Our research group also evaluated this association in a retrospective observational case series and demonstrated that the prevalence of *H. pylori* infection was 78.2% in CSC patients and 43.5% in control subjects^[117]. *H. pylori* eradication may lead to an improvement in CSC^[115,118,119]. In particular, Zavoloka *et al.*^[120] demonstrated that *H. pylori* eradication caused a decrease in the disease duration of 3 mo and in the recurrence frequency of 45.6 % and led to an improved long term prognosis. In fact, after two years, the visual acuity increased, the scotoma frequency decreased and the metamorphopsia frequency decreased.

Blepharitis is characterized by non-granulomatous inflammation of the eyelid margin. It remains difficult to ascertain whether its association with *H. pylori* infection is real because the data are highly discordant^[121,122].

CARDIOVASCULAR DISEASES

The association of *H. pylori* infection with cardiovascular diseases (CD) represents one of the fields of study that has attracted the most attention in the scientific community, despite the evident difficulties in identifying the specific role of *H. pylori* in the pathogenic processes of CD, including coronary atherosclerotic disease (CAD), stroke and myocardial infarction. Mendall *et al.*^[121] were the first to describe a significant association between *H. pylori* infection and the development of CAD in male subjects aged 45-65 years in a prospective study. CAD is due to dysfunction of the vessel endothelium associated with a simultaneous remodeling of the vessel wall, which is accompanied by an increase in blood pressure, a local inflammatory state and blood clotting; these are all phenomena that overall converge towards the formation of atherosclerotic plaques that are frequently unstable and susceptible to breakage. A possible rupture may compromise the blood circulation and lead to a myocardial infarction (MI). There are multiple causal factors that are involved in the pathogenesis during the initial stages of disease progression, including smoking, arterial hypertension, the presence of mixed dyslipidemia (alterations in total cholesterol levels, low density lipoprotein (LDL) and triglycerides) associated with a simultaneous reduction in high density lipoprotein

(HDL) cholesterol, obesity, diabetes mellitus, and the presence of hyperhomocysteinemia and increased coagulation factors^[123,124]. The hypothesis that infectious factors may play a role in the pathogenesis of CAD has developed only recently, probably stimulated by evidence that, despite the predisposing factor pattern, there is still a part of the population affected by CAD in which the origin and progression of the disease is inexplicable. Indeed, Lai *et al.*^[125] have shown that *H. pylori* infection increases the risk of acute coronary artery disease, even in the absence (or after the removal) of risk factors. The most likely pathogenic mechanism of the relationship between *H. pylori* and CAD is the initiation of a chronic inflammatory process associated with infection; however, it should be clarified if the role of *H. pylori* is to trigger the disease or only to accelerate its clinical course. The particular role of *H. pylori* in the pathogenesis of CAD is thought to be related to its ability to trigger a persistent chronic inflammatory state that is established within the gastric epithelium but that can also cause systemic inflammatory effects^[126]. In the stomach, VacA and urease contribute to the destruction of tight junctions, and this may allow the bacterial agents that breach the lamina propria to come into contact with the cells of the immune system^[127]. *H. pylori* antigens can either interact directly with the vascular endothelium or may form complexes with LDL/oxLDL cholesterol^[128]. The subacute inflammation present in chronic diseases, such as CAD, can be determined by the activation of the cells of the immune system or epithelial cells, through pattern recognition receptors (PRRs) that specifically recognize pathogen-associated molecular pattern^[129]. Both endothelial cells and macrophages present in atherosclerotic lesions show an overexpression of PRRs, such as TLR4, CD14 and TLR2, that are specialized for recognizing bacterial LPS^[130,131]. Xu *et al.*^[131] suggested a possible pathophysiological link between lipids, *H. pylori* infection, inflammatory status and CAD based on the overexpression of TLR4 on the surface of macrophages induced by pro-oxidant oxidized LDL. The inflammatory hypothesis was confirmed by several studies conducted with the intent of identifying an association between the increase in inflammatory markers and CAD. Li *et al.*^[132] and Libby *et al.*^[133] have highlighted a relationship between CAD and some of the most important inflammation markers, such as C-reactive protein (CRP), interleukin-6 (IL-6) and TNF- α , which are all expressed at higher levels in patients with atherosclerosis than in controls. CRP is an important acute phase protein that is associated with infectious and inflammatory processes or tissue damage; therefore, it is a reliable marker of endothelial dysfunction in CAD^[134,135]. A second hypothesis about the relationship between *H. pylori* infection and the pathogenesis of CAD is that bacterial antigens can induce T- and B-cell expansion and cause self-reactive antibody production by molecular mimicry. As an example of molecular mimicry, Matsuura *et al.*^[136] proposed that heat shock protein 60 derived from *H.*

pylori (Hp-HSP60) may potentially be related to the pathogenesis of CAD through the stimulation of Th1 lymphocytes, which are induced to produce INF- γ and IL-12, or through the activation of macrophages important in forming atherosclerotic plaques. An analysis of the literature clearly shows that serum positivity for CagA is closely associated with heart disease, including atherosclerosis, unstable angina, cardiac syndrome X and coronary artery disease^[137-139]. CagA is the most important virulence factor of *H. pylori*. CagA-positive strains are associated with increased IL-8 production, which is a marker of inflammation^[140]. The atherosclerotic cascade is notoriously characterized by an increase in the expression levels of inflammation markers, such as CRP and some interleukins. Discordant data about the association between *H. pylori* infection and CAD are present in the literature. In fact, Manolakis *et al.*^[141] did not find a correlation between CRP levels and *H. pylori* infection. Similarly, Carter *et al.*^[142] did not find any correlation between *H. pylori* infection and fibrinogen or von Willebrand's factor levels.

Discordant data about the relationship between *H. pylori* and myocardial infarction (MI) also exist. In 2013, Ikeda *et al.*^[143] demonstrated that only CagA-positive *H. pylori* strains were associated with an increased risk of MI. In a meta-analysis of 26000 patients, Liu *et al.*^[144] demonstrated the existence of a significant association between *H. pylori* infection and the risk of MI, whereas Hughes *et al.*^[145] showed a parallel decline of MI and duodenal ulcers in people born from 1930 to 1980. This study showed that the decline in MI was temporally related to a decline in duodenal ulcers and, by inference, *H. pylori* infection. The discordant data make it impossible to provide an unambiguous definition of *H. pylori* as a causal factor for cardiovascular diseases.

METABOLIC DISEASES

H. pylori infection has also been identified as related to alterations in glycolipid metabolism. The role of *H. pylori* infection in diabetes mellitus (DM), insulin resistance syndrome (IR), and metabolic syndrome (SMet) is still rather controversial, and in some cases, appears to bear marginal weight.

The association between *H. pylori* infection and DM has been suggested recently but still appears rather unclear^[146]. In a study of a Chinese population, Hsieh *et al.*^[147] demonstrated how high levels of glycated hemoglobin (HbA1c) were significantly associated with *H. pylori* infection in patients aged > 65 years. Bégué *et al.*^[148] demonstrated that eradication of *H. pylori* infection in patients with type 1 DM might be associated with better control of glycemia by evaluating HbA1c, which is an important indicator of long-term glycemic control, within two years after the eradication of the infection. In contrast, in 141 patients with type 2 DM, Demir *et al.*^[149] demonstrated no significant differences in either blood glucose levels or HbA1c values in patients

with *H. pylori* infections compared with controls. In a cross-sectional study including 1285 subjects aged 19-85 years, Yang *et al.*^[150] suggested the existence of a correlation between *H. pylori* infection and DM. According to Horikawa *et al.*^[151], the glycemic control of patients with DM was worse in the presence of *H. pylori* infections, in particular in infections with CagA-positive strains. A few studies demonstrated that eradication could lead to a benefit for glycemic control^[152,153]. This has not been confirmed by other studies^[154]. A meta-analysis by Wang *et al.*^[155] demonstrated the presence of higher levels of inflammatory markers in diabetic patients than in controls. Jeon *et al.*^[156] demonstrated that IL-6 levels and CRP levels, when used as inflammatory markers, appeared to be very similar in patients with DM, both in patients who were *H. pylori* positive and in those who were negative. As it is now known, chronic *H. pylori* infection is responsible for a low-level inflammatory state of the gastric mucosa, which is defined as the biological response of the mucosa to pathogens, that can involve different regions depending on the host phenotype. Chronic gastritis, such as that found in type 2 DM, atherosclerosis and SMet, is associated with an increase in circulating pro-inflammatory cytokine levels that are capable of interfering with both local and systemic metabolic processes^[157]. Studies have also been conducted evaluating complications associated with DM. Wang *et al.*^[158] described a possible correlation between *H. pylori* infection and the risk of nephropathy or neuropathy in an Asian population; however, some other authors did not confirm this correlation^[159]. Vafaeimanesh *et al.*^[160] have demonstrated the existence of an association between diabetic patients with microalbuminuria and *H. pylori* infection. Microalbuminuria, as well as neuropathy and heart disease, are very common complications in patients with DM, and patients with CagA-positive *H. pylori* infection are at a greater risk of developing these complications^[152,161].

Vafaeimanesh *et al.*^[160] also focused on the possible correlation between *H. pylori* infection and the development of IR. IR and *H. pylori* infection share pathogenic mediators that are potentially involved in the pathophysiology of the SMet^[161]. Zhou *et al.*^[162] showed that *H. pylori* may induce hepatic IR by interfering with the c-Jun/miR-203/SOCS3 pathway, both in humans and in animal models. An analysis of the literature shows that *H. pylori* eradication can improve IR, although this resolution seems to be associated with a progressive increase in BMI and cholesterol levels, as *H. pylori* suppresses ghrelin, which is the hormone responsible for increased appetite, which may explain the weight gain associated with the eradication of infection^[154]. However, one can hypothesize that the resolution of the symptoms related to infection may lead to recovery of a normal weight. HOMA-IR, which is a model for measuring insulin homeostasis, is higher in patients with *H. pylori* infection than in control^[163-165]. However, this finding has not been confirmed by other

studies^[166].

Considering the aforementioned role of *H. pylori* in IR, there have been several studies in the literature that demonstrate a higher prevalence of SMet in patients with *H. pylori* infection^[167]. Some studies have revealed changes in the lipid profiles of patients associated with the low-level inflammatory status induced by *H. pylori* infection; this has been demonstrated by Niemelä *et al.*^[168] in a study involving a Finnish population, in which serum cholesterol and triglyceride levels were higher in infected male subjects. Several studies have demonstrated the presence of these serum lipid profile alterations that can promote atherogenic processes^[169].

ALLERGIC DISEASES

In the literature, a number of studies and meta-analyses have reported an inverse association between *H. pylori* infection and asthma, worldwide^[170-172]. Blaser *et al.*^[173] showed the existence of an inverse relationship between *H. pylori* infection and the development of asthma or other allergic diseases, particularly in children and young people with an early onset of allergies. This relationship is controversial in adults, however, and has been confirmed mainly in cases in which the *H. pylori* strains are CagA-positive. Amberbir *et al.*^[174] showed that the relationship between *H. pylori* and rhinitis is not always inverse. *H. pylori* can almost completely protect against airway hyper-reactivity, broncho-alveolar eosinophilia, and lung inflammation, but this protection, which is strongly dependent on regulatory T (Treg) lymphocytes, is impaired by the complete eradication of the infection through antibiotic therapy^[170]. The functionality of T reg cells depends on IL-18 production by dendritic cells (DCs) following exposure to *H. pylori*, in the absence of which, a neonatal tolerance to infection is not established. Treg cells are derived from cells with an IL-18 -/- or an IL-18R -/- phenotype that have no protective capabilities against asthma; moreover, IL-18 is fundamental for the conversion of CD4+ T-cells into CD25+ Foxp3+ Treg cells^[175]. VacA toxin is the most important factor involved in protection against allergies. It appears to disrupt the T helper cell response to Treg cells. It stimulates Treg cells, but it also has the ability to interfere with antigen presentation and T-cell inhibition^[176]. *H. pylori* strains expressing the active form of VacA are usually also CagA positive^[177]. Most likely, CagA-positive strains elicit a greater response through modulation of inflammation by Treg cells, but it seems plausible that there is also a pronounced effect on the migration of Treg cells^[178]. To validate this hypothesis, Engler *et al.*^[179] demonstrated that the administration of VacA to mouse models was able to provide allergy protection that was comparable to that of active infection. However, these effects seem to be more pronounced if this administration is carried out during the neonatal stage of life, probably because this represents a crucial period during which antigenic

tolerance develops, both in mice and in humans. *H. pylori* influences the immune system by shifting the balance of cytokines to the Th1 type, which suppresses allergic diseases that are dependent on the Th2 cytotype^[180,181], protecting infected individuals from developing atopic disease. This might also explain the low prevalence of eosinophilic esophagitis in *H. pylori*-infected subjects^[182]. When analyzing the association between *H. pylori* infection and allergies one should take into account the hygiene hypothesis and the reduced prevalence of allergic diseases in pet owners. In fact, *H. pylori* infection is associated with poor hygiene, crowding and low socio-economic status. Poor hygiene conditions and low socioeconomic status together with pet ownership may expose to other bacteria or antigens which may reduce the risk of allergic diseases. Therefore, in the interpretation of epidemiologic studies between *H. pylori* infection and allergies, possible confounding factors should be considered before drawing conclusions on putative cause-effect relationship^[180-182].

HEPATOBIILIARY DISEASES

Recent studies have shown the involvement of *H. pylori* in the etiopathogenesis of a number of liver diseases^[183,184]. Polyzos *et al.*^[185] showed a higher serum level of anti-*H. pylori* IgG in patients with nonalcoholic fatty liver disease (NAFLD) than in non-NAFLD patients. Many other studies have shown an association between NAFLD and *H. pylori*^[186,187]. *H. pylori* could be related to a worsening of the inflammatory status of the liver, regardless of the etiology of the underlying liver disease^[184]. Fukuda *et al.*^[188] have proposed that because of the increase in gastric and intestinal mucosal permeability, *H. pylori* antigens could have access to the blood stream and reach the liver through the portal vein, thus causing liver damage. Some authors, such as Sumida *et al.*^[189], suggest that the eradication of *H. pylori* may play an important role in the treatment of nonalcoholic steatohepatitis (NASH), through the decrease of TNF α , one of the pro-inflammatory cytokines that, together with IL-1 β , IL-6 and IL-8, is also directly correlated with the etiopathogenesis of IR, and of NASH^[190,191]. Adiponectin is also involved in the etiopathogenesis of NAFLD. In fact adiponectin deficiency is associated with a pro-inflammatory condition, as it is observed in obesity and other metabolic disorders^[192]. According to Polyzos *et al.*^[185] a higher prevalence of low levels of circulating adiponectin, as well as of higher levels of anti-*H. pylori* IgG and elevated TNF α are observed in NAFLD patients compared to controls.

The correlation between *H. pylori* and hepatic fibrosis was analyzed mostly in animal models. Ki *et al.*^[193] demonstrated in a murine model that *H. pylori* infection may accelerate hepatic fibrosis through increased TGF- β 1-induced pro-inflammatory signaling pathways in hepatic stellate cells and that *H. pylori* infection might increase the risk of TGF- β 1-mediated tumorigenesis

[illegible]

Allergic diseases	Pro	Con
		Chen <i>et al</i> ^[170]
		Wang <i>et al</i> ^[171]
		Zhou <i>et al</i> ^[172]
		Blaser <i>et al</i> ^[173]
		Amberbir <i>et al</i> ^[174]
		Oertli <i>et al</i> ^[175]
		Oertli <i>et al</i> ^[176]
		Cook <i>et al</i> ^[178]
		Engler <i>et al</i> ^[179]
		Grad <i>et al</i> ^[180]
		Sheikh <i>et al</i> ^[181]
		Molina-Infante <i>et al</i> ^[182]

by disturbing the balance between apoptosis and proliferation of hepatocytes. Senescence marker protein-30, a protein that prevents oxidative stress and hepatic cell apoptosis^[194] is reduced in the liver of mice treated with CCL4 and *H. pylori* compared to those treated with CCL4 only^[195].

H. pylori has been considered as putative a triggering factor for autoimmune extragastric conditions^[196,197]. Goo *et al*^[198] in 2008 showed that C57BL/6 mouse infected with *H. pylori* developed a form of primary biliary cirrhosis (PBC) very similar to that described in humans. Because of the high serum levels of IgG against *H. pylori* VacA, the authors suggested that anti-VacA antibodies might play a role in the development of PBC. Shapira *et al*^[199] found higher prevalence of anti-*H. pylori* IgG in patients with PBC compared to controls. Nilsson *et al*^[200] evaluated 24 liver biopsies obtained from patients with PBC and primary sclerosing cholangitis (PSC) and found that *H. pylori* was present in 20/24 patients. The authors suggested that the pathogenic link between *H. pylori* infection and autoimmune liver diseases might be a form of molecular mimicry between *H. pylori* and liver antigens. In fact, a similar amino acid sequence homology between the main mitochondrial autoepitopic region from the E2 subunit of the pyruvate dehydrogenase complex and the *H. pylori* urease was found. However Bogdanos *et al*^[201] showed that the existence of this homology does not necessarily imply cross-reactivity. *H. pylori* DNA was detected in biliary epithelium in PSC patients compared to controls, corroborating the hypothesis that biliary reflux could lead to *H. pylori* contamination of the proximal biliary system, contributing to the development and / or progression of PSC in some patients^[202]. Patients with PSC may also suffer from ulcerative colitis (UC). It has therefore been suggested that an increased intestinal permeability in UC patients may promote the translocation of *H. pylori* into the hepatobiliary system, thus triggering autoimmunity mechanisms^[203].

The role of *H. pylori* in hepatic carcinogenesis is controversial. There are several known risk factors for HCC, such as alcoholism, PCB, PSC and chronic viral infections^[204]. The finding of *H. pylori* in hepatic biopsies of patients with HCC led to the hypothesis that *H. pylori* might be playing a role also in the development of this infection^[205]. In several studies, liver biopsies of

patients with HCC or even cholangiocarcinoma were positive for *H. pylori*^[206,207]. Dore *et al*^[208] demonstrated *H. pylori* presence in patients with HCC, liver cirrhosis and chronic hepatitis, using both PCR on liver tissue and serology. Prevalence of *H. pylori* infection was as high as 73% in patients with HCC. Verhoef *et al*^[209] and Pellicano *et al*^[210] also found positivity for *H. pylori* in 45% of patients with HCC vs 10% of controls, and in 85% of patients with HCC vs 33% of controls, respectively. Finally, it has been suggested that infection by CagA positive *H. pylori* strains might play a major role in the development of *H. pylori*-associated liver diseases^[211].

To date, there is no sufficient evidence to draw a firm conclusion on the relationship between *H. pylori* infection and liver diseases.

CONCLUSION

H. pylori is the cause of a number of gastroduodenal diseases including peptic ulcer disease and gastric adenocarcinoma which are the results of an interaction between bacterial virulence factors, host and environmental factors. Many extra-gastric manifestations have been reported to be linked to *H. pylori* infection (Table 1). However, most evidences derive from epidemiological studies where a number of confounding factors have not been analyzed in depth, thus making it impossible to establish a cause-effect correlation. As a result of this, to date, according to the last Consensus Report on the management of *H. pylori* infection (*i.e.*, Maastricht V/Florence Guidelines^[13]) *H. pylori* infection should be sought and, if present, eradicated only in patients with IDA, ITP and vitamin B12 deficiency.

REFERENCES

- 1 Mégraud F, Brassens-Rabbé MP, Denis F, Belbourni A, Hoa DQ. Seroepidemiology of Campylobacter pylori infection in various populations. *J Clin Microbiol* 1989; **27**: 1870-1873 [PMID: 2549098]
- 2 Go MF. Review article: natural history and epidemiology of Helicobacter pylori infection. *Aliment Pharmacol Ther* 2002; **16** Suppl 1: 3-15 [PMID: 11849122 DOI: 10.1046/j.1365-2036.2002.0160s1003.x]
- 3 Leclerc H. [Epidemiological aspects of Helicobacter pylori infection]. *Bull Acad Natl Med* 2006; **190**: 949-962 [PMID: 17195619]
- 4 Mandeville KL, Krabshuis J, Ladep NG, Mulder CJ, Quigley EM, Khan SA. Gastroenterology in developing countries: issues and advances. *World J Gastroenterol* 2009; **15**: 2839-2854 [PMID: 19533805 DOI: 10.3748/wjg.15.2839]
- 5 Ahmed KS, Khan AA, Ahmed I, Tiwari SK, Habeeb MA, Ali SM, Ahi JD, Abid Z, Alvi A, Hussain MA, Ahmed N, Habibullah CM. Prevalence study to elucidate the transmission pathways of Helicobacter pylori at oral and gastroduodenal sites of a South Indian population. *Singapore Med J* 2006; **47**: 291-296 [PMID: 16572240]
- 6 Mladenova I, Durazzo M. Transmission of Helicobacter pylori. *Minerva Gastroenterol Dietol* 2018; **64**: 251-254 [PMID: 29458239 DOI: 10.23736/S1121-421X.18.02480-7]
- 7 Blaser MJ. Hypothesis: the changing relationships of Helicobacter pylori and humans: implications for health and disease. *J Infect Dis*

- 1999; **179**: 1523-1530 [PMID: 10228075 DOI: 10.1086/314785]
- 8 **Lehours P**, Mégraud F. Helicobacter pylori infection and gastric MALT lymphoma. *Rocz Akad Med Białymst* 2005; **50**: 54-61 [PMID: 16358940]
 - 9 **Romano M**, Ricci V, Zarrilli R. Mechanisms of disease: Helicobacter pylori-related gastric carcinogenesis--implications for chemoprevention. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 622-632 [PMID: 17068500 DOI: 10.1038/ncpgasthep0634]
 - 10 **Censini S**, Stein M, Covacci A. Cellular responses induced after contact with Helicobacter pylori. *Curr Opin Microbiol* 2001; **4**: 41-46 [PMID: 11173032 DOI: 10.1016/S1369-5274(00)00162-4]
 - 11 **El-Omar EM**, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr, Rabkin CS. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 2000; **404**: 398-402 [PMID: 10746728 DOI: 10.1038/35006081]
 - 12 **Mendall MA**, Goggin PM, Molineaux N, Levy J, Toosy T, Strachan D, Camm AJ, Northfield TC. Relation of Helicobacter pylori infection and coronary heart disease. *Br Heart J* 1994; **71**: 437-439 [PMID: 8011406 DOI: 10.1136/hrt.71.5.437]
 - 13 **Malfertheiner P**, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus panel. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]
 - 14 **Chen Y**, Segers S, Blaser MJ. Association between Helicobacter pylori and mortality in the NHANES III study. *Gut* 2013; **62**: 1262-1269 [PMID: 23303440 DOI: 10.1136/gutjnl-2012-303018]
 - 15 **Wang ZW**, Li Y, Huang LY, Guan QK, Xu DW, Zhou WK, Zhang XZ. Helicobacter pylori infection contributes to high risk of ischemic stroke: evidence from a meta-analysis. *J Neurol* 2012; **259**: 2527-2537 [PMID: 22688569 DOI: 10.1007/s00415-012-6558-7]
 - 16 **Alvarez-Arellano L**, Maldonado-Bernal C. Helicobacter pylori and neurological diseases: Married by the laws of inflammation. *World J Gastrointest Pathophysiol* 2014; **5**: 400-404 [PMID: 25400983 DOI: 10.4291/wjgp.v5.i4.400]
 - 17 **Huang WS**, Yang TY, Shen WC, Lin CL, Lin MC, Kao CH. Association between Helicobacter pylori infection and dementia. *J Clin Neurosci* 2014; **21**: 1355-1358 [PMID: 24629396 DOI: 10.1016/j.jocn.2013.11.018]
 - 18 **Roubaud Baudron C**, Letenneur L, Langlais A, Buissonnière A, Mégraud F, Dartigues JF, Salles N; Personnes Agées QUID Study. Does Helicobacter pylori infection increase incidence of dementia? The Personnes Agées QUID Study. *J Am Geriatr Soc* 2013; **61**: 74-78 [PMID: 23252507 DOI: 10.1111/jgs.12065]
 - 19 **Beydoun MA**, Beydoun HA, Shroff MR, Kitner-Triolo MH, Zonderman AB. Helicobacter pylori seropositivity and cognitive performance among US adults: evidence from a large national survey. *Psychosom Med* 2013; **75**: 486-496 [PMID: 23697465 DOI: 10.1097/PSY.0b013e31829108c3]
 - 20 **Goni E**, Franceschi F. Helicobacter pylori and extragastric diseases. *Helicobacter* 2016; **21** Suppl 1: 45-48 [PMID: 27531539 DOI: 10.1111/hel.12340]
 - 21 **Kountouras J**, Boziki M, Gavalas E, Zavos C, Deretzi G, Chatzigeorgiou S, Katsinelos P, Grigoriadis N, Giartza-Taxidou E, Venizelos I. Five-year survival after Helicobacter pylori eradication in Alzheimer disease patients. *Cogn Behav Neurol* 2010; **23**: 199-204 [PMID: 20829670 DOI: 10.1097/WNN.0b013e3181df3034]
 - 22 **Kountouras J**, Boziki M, Gavalas E, Zavos C, Grigoriadis N, Deretzi G, Tzilves D, Katsinelos P, Tsolaki M, Chatzopoulos D, Venizelos I. Eradication of Helicobacter pylori may be beneficial in the management of Alzheimer's disease. *J Neurol* 2009; **256**: 758-767 [PMID: 19240960 DOI: 10.1007/s00415-009-5011-z]
 - 23 **Kountouras J**, Tsolaki F, Tsolaki M, Gavalas E, Zavos C, Polyzos SA, Boziki M, Katsinelos P, Kountouras C, Vardaka E, Tagarakis GI, Deretzi G. Helicobacter pylori-related ApoE 4 polymorphism may be associated with dysphagic symptoms in older adults. *Dis Esophagus* 2016; **29**: 842 [PMID: 25873443 DOI: 10.1111/dote.12364]
 - 24 **Santos CY**, Snyder PJ, Wu WC, Zhang M, Echeverria A, Alber J. Pathophysiologic relationship between Alzheimer's disease, cerebrovascular disease, and cardiovascular risk: A review and synthesis. *Alzheimers Dement* (Amst) 2017; **7**: 69-87 [PMID: 28275702 DOI: 10.1016/j.dadm.2017.01.005]
 - 25 **Kountouras J**, Boziki M, Gavalas E, Zavos C, Deretzi G, Grigoriadis N, Tsolaki M, Chatzopoulos D, Katsinelos P, Tzilves D, Zabouri A, Michailidou I. Increased cerebrospinal fluid Helicobacter pylori antibody in Alzheimer's disease. *Int J Neurosci* 2009; **119**: 765-777 [PMID: 19326283 DOI: 10.1080/00207450902782083]
 - 26 **Zelaya MV**, Pérez-Valderrama E, de Morentin XM, Tuñón T, Ferrer I, Luquin MR, Fernandez-Irigoyen J, Santamaría E. Olfactory bulb proteome dynamics during the progression of sporadic Alzheimer's disease: identification of common and distinct olfactory targets across Alzheimer-related co-pathologies. *Oncotarget* 2015; **6**: 39437-39456 [PMID: 26517091 DOI: 10.18632/oncotarget.6254]
 - 27 **Attems J**, Walker L, Jellinger KA. Olfactory bulb involvement in neurodegenerative diseases. *Acta Neuropathol* 2014; **127**: 459-475 [PMID: 24554308 DOI: 10.1007/s00401-014-1261-7]
 - 28 **Thomann PA**, Dos Santos V, Seidl U, Toro P, Essig M, Schröder J. MRI-derived atrophy of the olfactory bulb and tract in mild cognitive impairment and Alzheimer's disease. *J Alzheimers Dis* 2009; **17**: 213-221 [PMID: 19494444 DOI: 10.3233/JAD-2009-1036]
 - 29 **Förster S**, Vaitl A, Teipel SJ, Yakushev I, Mustafa M, la Fougère C, Rominger A, Cumming P, Bartenstein P, Hampel H, Hummel T, Buerger K, Hundt W, Steinbach S. Functional representation of olfactory impairment in early Alzheimer's disease. *J Alzheimers Dis* 2010; **22**: 581-591 [PMID: 20847402 DOI: 10.3233/JAD-2010-091549]
 - 30 **Doulberis M**, Kotronis G, Thomann R, Polyzos SA, Boziki M, Gialampirinou D, Deretzi G, Katsinelos P, Kountouras J. Review: Impact of Helicobacter pylori on Alzheimer's disease: What do we know so far? *Helicobacter* 2018; **23**: e12454 [PMID: 29181894 DOI: 10.1111/hel.12454]
 - 31 **Shiota S**, Murakami K, Yoshiiwa A, Yamamoto K, Ohno S, Kuroda A, Mizukami K, Hanada K, Okimoto T, Kodama M, Abe K, Yamaoka Y, Fujioka T. The relationship between Helicobacter pylori infection and Alzheimer's disease in Japan. *J Neurol* 2011; **258**: 1460-1463 [PMID: 21336779 DOI: 10.1007/s00415-011-5957-5]
 - 32 **Chang YP**, Chiu GF, Kuo FC, Lai CL, Yang YH, Hu HM, Chang PY, Chen CY, Wu DC, Yu FJ. Eradication of Helicobacter pylori Is Associated with the Progression of Dementia: A Population-Based Study. *Gastroenterol Res Pract* 2013; **2013**: 175729 [PMID: 24371435 DOI: 10.1155/2013/175729]
 - 33 **Mohebi N**, Mamarabadi M, Moghaddasi M. Relation of helicobacter pylori infection and multiple sclerosis in Iranian patients. *Neurol Int* 2013; **5**: 31-33 [PMID: 23888213 DOI: 10.4081/ni.2013.e10]
 - 34 **Franceschi F**, Gasbarrini A, Polyzos SA, Kountouras J. Extragastric Diseases and Helicobacter pylori. *Helicobacter* 2015; **20** Suppl 1: 40-46 [PMID: 26372824 DOI: 10.1111/hel.12256]
 - 35 **Cook KW**, Crooks J, Hussain K, O'Brien K, Braitch M, Kareem H, Constantinescu CS, Robinson K, Gran B. Helicobacter pylori infection reduces disease severity in an experimental model of multiple sclerosis. *Front Microbiol* 2015; **6**: 52 [PMID: 25762984 DOI: 10.3389/fmicb.2015.00052]
 - 36 **Shen X**, Yang H, Wu Y, Zhang D, Jiang H. Meta-analysis: Association of Helicobacter pylori infection with Parkinson's diseases. *Helicobacter* 2017; **22**: e12398 [PMID: 28598012 DOI: 10.1111/hel.12398]
 - 37 **Huang HK**, Wang JH, Lei WY, Chen CL, Chang CY, Liou LS. Helicobacter pylori infection is associated with an increased risk of Parkinson's disease: A population-based retrospective cohort study. *Parkinsonism Relat Disord* 2018; **47**: 26-31 [PMID: 29174171 DOI: 10.1016/j.parkreldis.2017.11.331]
 - 38 **Fasano A**, Bove F, Gabrielli M, Petracca M, Zocco MA, Ragazzoni E, Barbaro F, Piano C, Fortuna S, Tortora A, Di Giacomo R,

- Campanale M, Gigante G, Lauritano EC, Navarra P, Marconi S, Gasbarrini A, Bentivoglio AR. The role of small intestinal bacterial overgrowth in Parkinson's disease. *Mov Disord* 2013; **28**: 1241-1249 [PMID: 23712625 DOI: 10.1002/mds.25522]
- 39 **Tan AH**, Mahadeva S, Marras C, Thalha AM, Kiew CK, Yeat CM, Ng SW, Ang SP, Chow SK, Loke MF, Vadivelu JS, Ibrahim N, Yong HS, Tan CT, Fox SH, Lang AE, Lim SY. Helicobacter pylori infection is associated with worse severity of Parkinson's disease. *Parkinsonism Relat Disord* 2015; **21**: 221-225 [PMID: 25560322 DOI: 10.1016/j.parkreldis.2014.12.009]
- 40 **Mridula KR**, Borgohain R, Chandrasekhar Reddy V, Bandaru VCh, Suryaprabha T. Association of Helicobacter pylori with Parkinson's Disease. *J Clin Neurol* 2017; **13**: 181-186 [PMID: 28406585 DOI: 10.3988/jcn.2017.13.2.181]
- 41 **Candelario-Jalil E**, Taheri S, Yang Y, Sood R, Grossetete M, Estrada EY, Fiebich BL, Rosenberg GA. Cyclooxygenase inhibition limits blood-brain barrier disruption following intracerebral injection of tumor necrosis factor-alpha in the rat. *J Pharmacol Exp Ther* 2007; **323**: 488-498 [PMID: 17704356 DOI: 10.1124/jpet.107.127035]
- 42 **Lo YC**, Shih YT, Wu DC, Lee YC. In vitro effects of Helicobacter pylori-induced infection in gastric epithelial AGS cells on microglia-mediated toxicity in neuroblastoma SH-SY5Y cells. *Inflamm Res* 2009; **58**: 329-335 [PMID: 19247579 DOI: 10.1007/s00011-009-8075-4]
- 43 **Dobbs RJ**, Charlett A, Purkiss AG, Dobbs SM, Weller C, Peterson DW. Association of circulating TNF-alpha and IL-6 with ageing and parkinsonism. *Acta Neurol Scand* 1999; **100**: 34-41 [PMID: 10416510 DOI: 10.1111/j.1600-0404.1999.tb00721.x]
- 44 **Kountouras J**, Deretzi G, Zavos C, Karatzoglou P, Touloumis L, Nicolaides T, Chatzopoulos D, Venizelos I. Association between Helicobacter pylori infection and acute inflammatory demyelinating polyradiculoneuropathy. *Eur J Neurol* 2005; **12**: 139-143 [PMID: 15679702 DOI: 10.1111/j.1468-1331.2004.00977.x]
- 45 **Moran AP**, Prendergast MM. Molecular mimicry in Campylobacter jejuni and Helicobacter pylori lipopolysaccharides: contribution of gastrointestinal infections to autoimmunity. *J Autoimmun* 2001; **16**: 241-256 [PMID: 11334489 DOI: 10.1006/jaut.2000.0490]
- 46 **Chiba S**, Sugiyama T, Yonekura K, Tanaka S, Matsumoto H, Fujii N, Ebisu S, Sekiguchi K. An antibody to VacA of Helicobacter pylori in cerebrospinal fluid from patients with Guillain-Barre syndrome. *J Neurol Neurosurg Psychiatry* 2002; **73**: 76-78 [PMID: 12082053 DOI: 10.1136/jnnp.73.1.76]
- 47 **Gravina A**, Federico A, Ruocco E, Lo Schiavo A, Masarone M, Tuccillo C, Peccerillo F, Miranda A, Romano L, de Sio C, de Sio I, Persico M, Ruocco V, Riegler G, Loguercio C, Romano M. Helicobacter pylori infection but not small intestinal bacterial overgrowth may play a pathogenic role in rosacea. *United European Gastroenterol J* 2015; **3**: 17-24 [PMID: 25653855 DOI: 10.1177/2050640614559262]
- 48 **Argenziano G**, Donnarumma G, Iovene MR, Arnese P, Baldassarre MA, Baroni A. Incidence of anti-Helicobacter pylori and anti-CagA antibodies in rosacea patients. *Int J Dermatol* 2003; **42**: 601-604 [PMID: 12890101 DOI: 10.1046/j.1365-4362.2003.01817.x]
- 49 **El-Khalawany M**, Mahmoud A, Mosbeh AS, A B D Alsalam F, Ghonaim N, Abou-Bakr A. Role of Helicobacter pylori in common rosacea subtypes: a genotypic comparative study of Egyptian patients. *J Dermatol* 2012; **39**: 989-995 [PMID: 23039081 DOI: 10.1111/j.1346-8138.2012.01675.x]
- 50 **Campanati A**, Ganzetti G, Martina E, Giannoni M, Gesuita R, Bendia E, Giuliadori K, Sandroni L, Offidani A. Helicobacter pylori infection in psoriasis: results of a clinical study and review of the literature. *Int J Dermatol* 2015; **54**: e109-e114 [PMID: 25808243 DOI: 10.1111/ijd.12798]
- 51 **Azizzadeh M**, Nejad ZV, Ghorbani R, Pahlevan D. Relationship between Helicobacter pylori infection and psoriasis. *Ann Saudi Med* 2014; **34**: 241-244 [PMID: 25266185 DOI: 10.5144/0256-4947.2014.241]
- 52 **Qayoom S**, Ahmad QM. Psoriasis and Helicobacter pylori. *Indian J Dermatol Venereol Leprol* 2003; **69**: 133-134 [PMID: 17642857]
- 53 **Mesquita PM**, Diogo A Filho, Jorge MT, Berbert AL, Mantese SA, Rodrigues JJ. Relationship of Helicobacter pylori seroprevalence with the occurrence and severity of psoriasis. *An Bras Dermatol* 2017; **92**: 52-57 [PMID: 28225957 DOI: 10.1590/abd1806-4841.20174880]
- 54 **Mesquita PM**, Diogo A Filho, Jorge MT, Berbert ALCV, Mantese SA, Rodrigues JJ. Comment on Helicobacter pylori seroprevalence and the occurrence and severity of psoriasis - Reply. *An Bras Dermatol* 2017; **92**: 585 [PMID: 28954123 DOI: 10.1590/abd1806-4841.20177256]
- 55 **Onsun N**, Arda Ulusal H, Su O, Beycan I, Biyik Ozkaya D, Senocak M. Impact of Helicobacter pylori infection on severity of psoriasis and response to treatment. *Eur J Dermatol* 2012; **22**: 117-120 [PMID: 22063790 DOI: 10.1684/ejd.2011.1579]
- 56 **Hizal M**, Tüzün B, Wolf R, Tüzün Y. The relationship between Helicobacter pylori IgG antibody and autologous serum test in chronic urticaria. *Int J Dermatol* 2000; **39**: 443-445 [PMID: 10944089 DOI: 10.1046/j.1365-4362.2000.00979.x]
- 57 **Galadari IH**, Sheriff MO. The role of Helicobacter pylori in urticaria and atopic dermatitis. *Skinmed* 2006; **5**: 172-176 [PMID: 16855407 DOI: 10.1111/j.1540-9740.2006.04646.x]
- 58 **Campanati A**, Gesuita R, Giannoni M, Piraccini F, Sandroni L, Martina E, Conocchiarì L, Bendia E, Di Sario A, Offidani A. Role of small intestinal bacterial overgrowth and Helicobacter pylori infection in chronic spontaneous urticaria: a prospective analysis. *Acta Derm Venereol* 2013; **93**: 161-164 [PMID: 22858910 DOI: 10.2340/00015555-1373]
- 59 **Yoshimasu T**, Furukawa F. Eradication therapy for urticaria with high titers of anti H. pylori IgG antibody. *Allergol Int* 2014; **63**: 37-40 [PMID: 24270226 DOI: 10.2332/allergolint.13-OA-0580]
- 60 **Rigopoulos D**, Katsambas A, Karalexis A, Papatheodorou G, Rokkas T. No increased prevalence of Helicobacter pylori in patients with alopecia areata. *J Am Acad Dermatol* 2002; **46**: 141 [PMID: 11756964 DOI: 10.1067/mjd.2002.117255]
- 61 **Behrangi E**, Mansouri P, Agah S, Ebrahimi Daryani N, Mokhtare M, Azizi Z, Ramezani Ghamsari M, Rohani Nasab M, Azizian Z. Association between Helicobacter Pylori Infection and Alopecia Areata: A Study in Iranian Population. *Middle East J Dig Dis* 2017; **9**: 107-110 [PMID: 28638587 DOI: 10.15171/mejdd.2017.59]
- 62 **Ljubojevic S**, Lipozenčić J. Autoimmune bullous diseases associations. *Clin Dermatol* 2012; **30**: 17-33 [PMID: 22137223 DOI: 10.1016/j.clindermatol.2011.03.006]
- 63 **Magen E**, Delgado JS. Helicobacter pylori and skin autoimmune diseases. *World J Gastroenterol* 2014; **20**: 1510-1516 [PMID: 24587626 DOI: 10.3748/wjg.v20.i6.1510]
- 64 **Sagi L**, Baum S, Agmon-Levin N, Sherer Y, Katz BS, Barzilai O, Ram M, Bizzaro N, SanMarco M, Trau H, Shoenfeld Y. Autoimmune bullous diseases the spectrum of infectious agent antibodies and review of the literature. *Autoimmun Rev* 2011; **10**: 527-535 [PMID: 21527361 DOI: 10.1016/j.autrev.2011.04.003]
- 65 **Mortazavi H**, Hejazi P, Khamesipour A, Mohebbi M, Ehsani AH, Mohammadi Y, Farahani IV, Amirzargar AA. Frequency of seropositivity against infectious agents amongst pemphigus vulgaris patients: a case-control study on Strongyloides stercoralis, Helicobacter pylori, Toxoplasma gondii, Leishmania major, and Epstein-Barr virus. *Int J Dermatol* 2015; **54**: e458-e465 [PMID: 26175264 DOI: 10.1111/ijd.12869]
- 66 **Novák J**, Szekanecz Z, Sebesi J, Takáts A, Demeter P, Bene L, Sipka S, Csiki Z. Elevated levels of anti-Helicobacter pylori antibodies in Henoch-Schönlein purpura. *Autoimmunity* 2003; **36**: 307-311 [PMID: 14567560 DOI: 10.1080/08916930232000114535]
- 67 **Grivceva-Panovska V**, Grivceva Stardelova K, Serafimovski V. Henoch-Schönlein purpura in an adult patient: extragastric, cutaneous manifestation of helicobacter pylori infection. *Prilozi* 2008; **29**: 291-301 [PMID: 18709017]
- 68 **Hoshino C**. Adult onset Schönlein-Henoch purpura associated with Helicobacter pylori infection. *Intern Med* 2009; **48**: 847-851 [PMID: 19443983 DOI: 10.2169/internalmedicine.48.1718]
- 69 **Blecker U**, Renders F, Lanciers S, Vandenplas Y. Syncope leading to the diagnosis of a Helicobacter pylori positive chronic active

- haemorrhagic gastritis. *Eur J Pediatr* 1991; **150**: 560-561 [PMID: 1954961 DOI: 10.1007/BF02072207]
- 70 **Muhsen K**, Cohen D. Helicobacter pylori infection and iron stores: a systematic review and meta-analysis. *Helicobacter* 2008; **13**: 323-340 [PMID: 19250507 DOI: 10.1111/j.1523-5378.2008.00617.x]
 - 71 **Qu XH**, Huang XL, Xiong P, Zhu CY, Huang YL, Lu LG, Sun X, Rong L, Zhong L, Sun DY, Lin H, Cai MC, Chen ZW, Hu B, Wu LM, Jiang YB, Yan WL. Does Helicobacter pylori infection play a role in iron deficiency anemia? A meta-analysis. *World J Gastroenterol* 2010; **16**: 886-896 [PMID: 20143469 DOI: 10.3748/wjg.v16.i7.886]
 - 72 **Huang X**, Qu X, Yan W, Huang Y, Cai M, Hu B, Wu L, Lin H, Chen Z, Zhu C, Lu L, Sun X, Rong L, Jiang Y, Sun D, Zhong L, Xiong P. Iron deficiency anaemia can be improved after eradication of Helicobacter pylori. *Postgrad Med J* 2010; **86**: 272-278 [PMID: 20448223 DOI: 10.1136/pgmj.2009.089987]
 - 73 **Yuan W**, Li Yumin, Yang Kehu, Ma Bin, Guan Quanlin, Wang D, Yang L. Iron deficiency anemia in Helicobacter pylori infection: meta-analysis of randomized controlled trials. *Scand J Gastroenterol* 2010; **45**: 665-676 [PMID: 20201716 DOI: 10.3109/00365521003663670]
 - 74 **Zhang ZF**, Yang N, Zhao G, Zhu L, Zhu Y, Wang LX. Effect of Helicobacter pylori eradication on iron deficiency. *Chin Med J (Engl)* 2010; **123**: 1924-1930 [PMID: 20819579]
 - 75 **Azab SF**, Esh AM. Serum hepcidin levels in Helicobacter pylori-infected children with iron-deficiency anemia: a case-control study. *Ann Hematol* 2013; **92**: 1477-1483 [PMID: 23760782 DOI: 10.1007/s00277-013-1813-2]
 - 76 **Kroot JJ**, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: diagnostic implications. *Clin Chem* 2011; **57**: 1650-1669 [PMID: 21989113 DOI: 10.1373/clinchem.2009.140053]
 - 77 **Ozkasap S**, Yerali N, Isik P, Bay A, Kara A, Tunc B. The role of prohepcidin in anemia due to Helicobacter pylori infection. *Pediatr Hematol Oncol* 2013; **30**: 425-431 [PMID: 23560993 DOI: 10.3109/08880018.2013.783144]
 - 78 **Senkovich O**, Ceaser S, McGee DJ, Testerman TL. Unique host iron utilization mechanisms of Helicobacter pylori revealed with iron-deficient chemically defined media. *Infect Immun* 2010; **78**: 1841-1849 [PMID: 20176792 DOI: 10.1128/IAI.01258-09]
 - 79 **Yokota S**, Toita N, Yamamoto S, Fujii N, Konno M. Positive relationship between a polymorphism in Helicobacter pylori neutrophil-activating protein a gene and iron-deficiency anemia. *Helicobacter* 2013; **18**: 112-116 [PMID: 23067298 DOI: 10.1111/hel.12011]
 - 80 **Testerman TL**, Morris J. Beyond the stomach: an updated view of Helicobacter pylori pathogenesis, diagnosis, and treatment. *World J Gastroenterol* 2014; **20**: 12781-12808 [PMID: 25278678 DOI: 10.3748/wjg.v20.i36.12781]
 - 81 **Affifi MT**, Abd El-Aziz HK, Hamed NA, Barghash NA, Abdo A, Gamal M. Role of Helicobacter pylori in refractory iron deficiency anaemia. *Br J Biomed Sci* 2009; **66**: 133-136 [PMID: 19839223 DOI: 10.1080/09674845.2009.11730259]
 - 82 **Hershko C**, Camaschella C. How I treat unexplained refractory iron deficiency anemia. *Blood* 2014; **123**: 326-333 [PMID: 24215034 DOI: 10.1182/blood-2013-10-512624]
 - 83 **Hershko C**, Ronson A. Iron deficiency, Helicobacter infection and gastritis. *Acta Haematol* 2009; **122**: 97-102 [PMID: 19907146 DOI: 10.1159/000243793]
 - 84 **Kang JM**, Kim N, Lee BH, Park HK, Jo HJ, Shin CM, Lee SH, Park YS, Hwang JH, Kim JW, Jeong SH, Lee DH, Jung HC, Song IS. Risk factors for peptic ulcer bleeding in terms of Helicobacter pylori, NSAIDs, and antiplatelet agents. *Scand J Gastroenterol* 2011; **46**: 1295-1301 [PMID: 21815866 DOI: 10.3109/00365521.2011.605468]
 - 85 **Musumba C**, Jorgensen A, Sutton L, Van Eker D, Moorcroft J, Hopkins M, Pritchard DM, Pirmohamed M. The relative contribution of NSAIDs and Helicobacter pylori to the aetiology of endoscopically-diagnosed peptic ulcer disease: observations from a tertiary referral hospital in the UK between 2005 and 2010. *Aliment Pharmacol Ther* 2012; **36**: 48-56 [PMID: 22554233 DOI: 10.1111/j.1365-2036.2012.05118.x]
 - 86 **De Leest HT**, Steen KS, Bloemena E, Lems WF, Kuipers EJ, Van de Laar MA, Bijlsma JW, Janssen M, Houben HH, Kostense PJ, Boers M, Dijkmans BA. Helicobacter pylori eradication in patients on long-term treatment with NSAIDs reduces the severity of gastritis: a randomized controlled trial. *J Clin Gastroenterol* 2009; **43**: 140-146 [PMID: 18797408 DOI: 10.1097/MCG.0b013e3181595b40]
 - 87 **Campuzano-Maya G**. Hematologic manifestations of Helicobacter pylori infection. *World J Gastroenterol* 2014; **20**: 12818-12838 [PMID: 25278680 DOI: 10.3748/wjg.v20.i36.12818]
 - 88 **Lahner E**, Persechini S, Annibale B. Micronutrients (Other than iron) and Helicobacter pylori infection: a systematic review. *Helicobacter* 2012; **17**: 1-15 [PMID: 22221610 DOI: 10.1111/j.1523-5378.2011.00892.x]
 - 89 **Andrés E**, Loukili NH, Noel E, Kaltenbach G, Abdelgheni MB, Perrin AE, Noblet-Dick M, Maloisel F, Schlienger JL, Blicklé JF. Vitamin B12 (cobalamin) deficiency in elderly patients. *CMAJ* 2004; **171**: 251-259 [PMID: 15289425 DOI: 10.1503/cmaj.1031155]
 - 90 **Marino MC**, de Oliveira CA, Rocha AM, Rocha GA, Clementino NC, Antunes LF, Oliveira RA, Martins AS, Del Puerto HL, D'Almeida V, Galdieri L, Pedroso ER, Cabral MM, Nogueira AM, Queiroz DM. Long-term effect of Helicobacter pylori eradication on plasma homocysteine in elderly patients with cobalamin deficiency. *Gut* 2007; **56**: 469-474 [PMID: 17005765 DOI: 10.1136/gut.2006.095125]
 - 91 **Toh BH**, van Driel IR, Gleeson PA. Pernicious anemia. *N Engl J Med* 1997; **337**: 1441-1448 [PMID: 9358143 DOI: 10.1056/NEJM199711133372007]
 - 92 **García Pérez A**, Valverde de La Osa J, Giménez Samper M, Alonso García I. Resolution of an autoimmune thrombocytopenic purpura after eradicating treatment of Helicobacter pylori. *Sangre (Barc)* 1999; **44**: 387-388 [PMID: 10618919]
 - 93 **Stasi R**, Rossi Z, Stipa E, Amadori S, Newland AC, Provan D. Helicobacter pylori eradication in the management of patients with idiopathic thrombocytopenic purpura. *Am J Med* 2005; **118**: 414-419 [PMID: 15808140 DOI: 10.1016/j.amjmed.2004.09.014]
 - 94 **Gasbarrini A**, Franceschi F, Tartaglione R, Landolfi R, Pola P, Gasbarrini G. Regression of autoimmune thrombocytopenia after eradication of Helicobacter pylori. *Lancet* 1998; **352**: 878 [PMID: 9742983 DOI: 10.1016/S0140-6736(05)60004-9]
 - 95 **Suvajdzic N**, Stanković B, Artiko V, Cvejić T, Bulat V, Bakrac M, Colović M, Obradović V, Atkinson HD. Helicobacter pylori eradication can induce platelet recovery in chronic idiopathic thrombocytopenic purpura. *Platelets* 2006; **17**: 227-230 [PMID: 16769600 DOI: 10.1080/09537100500462487]
 - 96 **Jackson SC**, Beck P, Buret AG, O'Connor PM, Meddings J, Pineo G, Poon MC. Long term platelet responses to Helicobacter pylori eradication in Canadian patients with immune thrombocytopenic purpura. *Int J Hematol* 2008; **88**: 212-218 [PMID: 18668306 DOI: 10.1007/s12185-008-0138-8]
 - 97 **Jarque I**, Andreu R, Llopis I, De la Rubia J, Gomis F, Senent L, Jiménez C, Martín G, Martínez JA, Sanz GF, Ponce J, Sanz MA. Absence of platelet response after eradication of Helicobacter pylori infection in patients with chronic idiopathic thrombocytopenic purpura. *Br J Haematol* 2001; **115**: 1002-1003 [PMID: 11843840 DOI: 10.1046/j.1365-2141.2001.03194.x]
 - 98 **Michel M**, Khellaf M, Desforages L, Lee K, Schaeffer A, Godeau B, Bierling P. Autoimmune thrombocytopenic Purpura and Helicobacter pylori infection. *Arch Intern Med* 2002; **162**: 1033-1036 [PMID: 11996614 DOI: 10.1001/archinte.162.9.1033]
 - 99 **Michel M**, Cooper N, Jean C, Frissora C, Bussel JB. Does Helicobacter pylori initiate or perpetuate immune thrombocytopenic purpura? *Blood* 2004; **103**: 890-896 [PMID: 12920031 DOI: 10.1182/blood-2003-03-0900]
 - 100 **Estrada-Gómez RA**, Parra-Ortega I, Martínez-Barreda C, Ruiz-Argüelles GJ. Helicobacter pylori infection and thrombocytopenia:

- a single-institution experience in Mexico. *Rev Invest Clin* 2007; **59**: 112-115 [PMID: 17633798]
- 101 **Satoh T**, Pandey JP, Okazaki Y, Asahi A, Kawakami Y, Ikeda Y, Kuwana M. Single nucleotide polymorphism of interleukin-1beta associated with *Helicobacter pylori* infection in immune thrombocytopenic purpura. *Tissue Antigens* 2009; **73**: 353-357 [PMID: 19317746 DOI: 10.1111/j.1399-0039.2009.01214.x]
- 102 **Asahi A**, Nishimoto T, Okazaki Y, Suzuki H, Masaoka T, Kawakami Y, Ikeda Y, Kuwana M. *Helicobacter pylori* eradication shifts monocyte Fcgamma receptor balance toward inhibitory FcgammaRIIB in immune thrombocytopenic purpura patients. *J Clin Invest* 2008; **118**: 2939-2949 [PMID: 18654664 DOI: 10.1172/JCI34496]
- 103 **Kodama M**, Kitadai Y, Ito M, Kai H, Masuda H, Tanaka S, Yoshihara M, Fujimura K, Chayama K. Immune response to CagA protein is associated with improved platelet count after *Helicobacter pylori* eradication in patients with idiopathic thrombocytopenic purpura. *Helicobacter* 2007; **12**: 36-42 [PMID: 17241299 DOI: 10.1111/j.1523-5378.2007.00477.x]
- 104 **Byrne MF**, Kerrigan SW, Corcoran PA, Atherton JC, Murray FE, Fitzgerald DJ, Cox DM. *Helicobacter pylori* binds von Willebrand factor and interacts with GPIb to induce platelet aggregation. *Gastroenterology* 2003; **124**: 1846-1854 [PMID: 12806618 DOI: 10.1016/S0016-5085(03)00397-4]
- 105 **Cicconi V**, Carloni E, Franceschi F, Nocente R, Silveri NG, Manna R, Servidei S, Bentivoglio AR, Gasbarrini A, Gasbarrini G. Disappearance of antiphospholipid antibodies syndrome after *Helicobacter pylori* eradication. *Am J Med* 2001; **111**: 163-164 [PMID: 11501549 DOI: 10.1016/S0002-9343(01)00738-0]
- 106 **Lim W**, Crowther MA, Eikelboom JW. Management of antiphospholipid antibody syndrome: a systematic review. *JAMA* 2006; **295**: 1050-1057 [PMID: 16507806 DOI: 10.1001/jama.295.9.1050]
- 107 **Papadaki HA**, Pontikoglou C, Stavroulaki E, Minadakis G, Eliopoulos DA, Pyrovolaki K, Skordilis P, Eliopoulos GD. High prevalence of *Helicobacter pylori* infection and monoclonal gammopathy of undetermined significance in patients with chronic idiopathic neutropenia. *Ann Hematol* 2005; **84**: 317-320 [PMID: 15731921 DOI: 10.1007/s00277-004-0996-y]
- 108 **Papadaki HA**, Pontikoglou C, Eliopoulos DG, Pyrovolaki K, Spyridaki R, Eliopoulos GD. *Helicobacter pylori* infection is probably the cause of chronic idiopathic neutropenia (CIN)-associated splenomegaly. *Am J Hematol* 2006; **81**: 142-144 [PMID: 16432851 DOI: 10.1002/ajh.20496]
- 109 **Tursi A**, Modeo ME. Monoclonal gammopathy of undetermined significance predisposing to *Helicobacter pylori*-related gastric mucosa-associated lymphoid tissue lymphoma. *J Clin Gastroenterol* 2002; **34**: 147-149 [PMID: 11782609 DOI: 10.1097/00004836-200202000-00009]
- 110 **Wolkersdörfer GW**, Haase M, Morgner A, Baretton G, Miehlke S. Monoclonal gammopathy of undetermined significance and Russell body formation in *Helicobacter pylori* gastritis. *Helicobacter* 2006; **11**: 506-510 [PMID: 16961813 DOI: 10.1111/j.1523-5378.2006.00443.x]
- 111 **Rajkumar SV**, Kyle RA, Plevak MF, Murray JA, Therneau TM. *Helicobacter pylori* infection and monoclonal gammopathy of undetermined significance. *Br J Haematol* 2002; **119**: 706-708 [PMID: 12437647 DOI: 10.1046/j.1365-2141.2002.03912.x]
- 112 **Galloway PH**, Warner SJ, Morshed MG, Mikelberg FS. *Helicobacter pylori* infection and the risk for open-angle glaucoma. *Ophthalmology* 2003; **110**: 922-925 [PMID: 12750090 DOI: 10.1016/S0161-6420(03)00093-9]
- 113 **Kurtz S**, Regenbogen M, Goldiner I, Horowitz N, Moshkowitz M. No association between *Helicobacter pylori* infection or CagA-bearing strains and glaucoma. *J Glaucoma* 2008; **17**: 223-226 [PMID: 18414109 DOI: 10.1097/IJG.0b013e31815a34ac]
- 114 **Zeng J**, Liu H, Liu X, Ding C. The Relationship Between *Helicobacter pylori* Infection and Open-Angle Glaucoma: A Meta-Analysis. *Invest Ophthalmol Vis Sci* 2015; **56**: 5238-5245 [PMID: 26258610 DOI: 10.1167/iovs.15-17059]
- 115 **Casella AM**, Berbel RF, Bressanim GL, Malaguido MR, Cardillo JA. *Helicobacter pylori* as a potential target for the treatment of central serous chorioretinopathy. *Clinics* (Sao Paulo) 2012; **67**: 1047-1052 [PMID: 23018302 DOI: 10.6061/clinics/2012(09)11]
- 116 **Liu B**, Deng T, Zhang J. RISK FACTORS FOR CENTRAL SEROUS CHORIORETINOPATHY: A Systematic Review and Meta-Analysis. *Retina* 2016; **36**: 9-19 [PMID: 26710181 DOI: 10.1097/IAE.0000000000000837]
- 117 **Cotticelli L**, Borrelli M, D'Alessio AC, Menzione M, Villani A, Piccolo G, Montella F, Iovene MR, Romano M. Central serous chorioretinopathy and *Helicobacter pylori*. *Eur J Ophthalmol* 2006; **16**: 274-278 [PMID: 16703546 DOI: 10.1177/112067210601600213]
- 118 **Rahbani-Nobar MB**, Javadzadeh A, Ghojzadeh L, Rafeey M, Ghorbanihaghjo A. The effect of *Helicobacter pylori* treatment on remission of idiopathic central serous chorioretinopathy. *Mol Vis* 2011; **17**: 99-103 [PMID: 21245962]
- 119 **Dang Y**, Mu Y, Zhao M, Li L, Guo Y, Zhu Y. The effect of eradicating *Helicobacter pylori* on idiopathic central serous chorioretinopathy patients. *Ther Clin Risk Manag* 2013; **9**: 355-360 [PMID: 24043941 DOI: 10.2147/TCRM.S50407]
- 120 **Zavoloka O**, Bezditko P, Lahorzhevskaya I, Zubkova D, Ilyina Y. Clinical efficiency of *Helicobacter pylori* eradication in the treatment of patients with acute central serous chorioretinopathy. *Graefes Arch Clin Exp Ophthalmol* 2016; **254**: 1737-1742 [PMID: 26979068 DOI: 10.1007/s00417-016-3315-0]
- 121 **Saccà SC**, Vagge A, Pulliero A, Izzotti A. *Helicobacter pylori* infection and eye diseases: a systematic review. *Medicine* (Baltimore) 2014; **93**: e216 [PMID: 25526440 DOI: 10.1097/MD.0000000000000216]
- 122 **Saccà SC**, Pascotto A, Venturino GM, Prigione G, Mastromarino A, Baldi F, Bilardi C, Savarino V, Brusati C, Rebora A. Prevalence and treatment of *Helicobacter pylori* in patients with blepharitis. *Invest Ophthalmol Vis Sci* 2006; **47**: 501-508 [PMID: 16431942 DOI: 10.1167/iovs.05-0323]
- 123 **Linz B**, Balloux F, Moodley Y, Manica A, Liu H, Roumagnac P, Falush D, Stamer C, Prugnolle F, van der Merwe SW, Yamaoka Y, Graham DY, Perez-Trallero E, Wadstrom T, Suerbaum S, Achtman M. An African origin for the intimate association between humans and *Helicobacter pylori*. *Nature* 2007; **445**: 915-918 [PMID: 17287725 DOI: 10.1038/nature05562]
- 124 **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315 [PMID: 6145023 DOI: 10.1016/S0140-6736(84)91816-6]
- 125 **Lai CY**, Yang TY, Lin CL, Kao CH. *Helicobacter pylori* infection and the risk of acute coronary syndrome: a nationwide retrospective cohort study. *Eur J Clin Microbiol Infect Dis* 2015; **34**: 69-74 [PMID: 25063740 DOI: 10.1007/s10096-014-2207-7]
- 126 **Chmiela M**, Gajewski A, Rudnicka K. *Helicobacter pylori* vs coronary heart disease - searching for connections. *World J Cardiol* 2015; **7**: 187-203 [PMID: 25914788 DOI: 10.4330/wjc.v7.i4.187]
- 127 **Wroblewski LE**, Peek RM Jr. Targeted disruption of the epithelial-barrier by *Helicobacter pylori*. *Cell Commun Signal* 2011; **9**: 29 [PMID: 22044698 DOI: 10.1186/1478-811X-9-29]
- 128 **Chmiela M**, Miszczyk E, Rudnicka K. Structural modifications of *Helicobacter pylori* lipopolysaccharide: an idea for how to live in peace. *World J Gastroenterol* 2014; **20**: 9882-9897 [PMID: 25110419 DOI: 10.3748/wjg.v20.i29.9882]
- 129 **Chalubinski M**, Wojdan K, Dorantowicz R, Jackowska P, Gorzelak P, Broncel M. Comprehensive insight into immune regulatory mechanisms and vascular wall determinants of atherogenesis - emerging perspectives of immunomodulation. *Arch Med Sci* 2013; **9**: 159-165 [PMID: 23515919 DOI: 10.5114/aoms.2013.33355]
- 130 **Libby P**. Inflammation in atherosclerosis. *Nature* 2002; **420**: 868-874 [PMID: 12490960 DOI: 10.1038/nature01323]
- 131 **Xu XH**, Shah PK, Faure E, Equils O, Thomas L, Fishbein MC, Luthringer D, Xu XP, Rajavashisth TB, Yano J, Kaul S, Arditi M. Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. *Circulation* 2001; **104**: 3103-3108 [PMID: 11748108 DOI: 10.1161/hc5001.100631]

- 132 **Li J**, Li JJ, Li Q, Li Z, Qian HY. A rational connection of inflammation with peripheral arterial disease. *Med Hypotheses* 2007; **69**: 1190-1195 [PMID: 17555883 DOI: 10.1016/j.mehy.2007.02.043]
- 133 **Libby P**, Ridker PM, Hansson GK, Leducq Transatlantic Network on Atherothrombosis. Inflammation in atherosclerosis: from pathophysiology to practice. *J Am Coll Cardiol* 2009; **54**: 2129-2138 [PMID: 19942084 DOI: 10.1016/j.jacc.2009.09.009]
- 134 **Perkov S**, Paro MMK, Vidjak V, Flegar-Mestric Z. The Evaluation of New Biomarkers of Inflammation and Angiogenesis in Peripheral Arterial Disease. In: Rezzani R, editor. Current trends in atherogenesis. London: IntechOpen, 2013: 97-120 [DOI: 10.5772/53341]
- 135 **Vahdat K**, Jafari SM, Pazoki R, Nabipour I. Concurrent increased high sensitivity C-reactive protein and chronic infections are associated with coronary artery disease: a population-based study. *Indian J Med Sci* 2007; **61**: 135-143 [PMID: 17337814 DOI: 10.4103/0019-5359.30748]
- 136 **Matsuura E**, Kobayashi K, Matsunami Y, Shen L, Quan N, Makarova M, Suchkov SV, Ayada K, Oguma K, Lopez LR. Autoimmunity, infectious immunity, and atherosclerosis. *J Clin Immunol* 2009; **29**: 714-721 [PMID: 19795194 DOI: 10.1007/s10875-009-9333-5]
- 137 **Khodaii Z**, Vakili H, Ghaderian SM, Najari RA, Panah AS. Association of Helicobacter pylori infection with acute myocardial infarction. *Coron Artery Dis* 2011; **22**: 6-11 [PMID: 20962628 DOI: 10.1097/MCA.0b013e3283402360]
- 138 **Rasmi Y**, Raeisi S, Seyyed Mohammadzad MH. Association of inflammation and cytotoxin-associated gene a positive strains of helicobacter pylori in cardiac syndrome x. *Helicobacter* 2012; **17**: 116-120 [PMID: 22404441 DOI: 10.1111/j.1523-5378.2011.00923.x]
- 139 **Zhang S**, Guo Y, Ma Y, Teng Y. Cytotoxin-associated gene-A-seropositive virulent strains of Helicobacter pylori and atherosclerotic diseases: a systematic review. *Chin Med J (Engl)* 2008; **121**: 946-951 [PMID: 18706211]
- 140 **Fischer W**, Prassl S, Haas R. Virulence mechanisms and persistence strategies of the human gastric pathogen Helicobacter pylori. *Curr Top Microbiol Immunol* 2009; **337**: 129-171 [PMID: 19812982 DOI: 10.1007/978-3-642-01846-6_5]
- 141 **Manolakis A**, Kapsoritakis AN, Potamianos SP. A review of the postulated mechanisms concerning the association of Helicobacter pylori with ischemic heart disease. *Helicobacter* 2007; **12**: 287-297 [PMID: 17669100 DOI: 10.1111/j.1523-5378.2007.00511.x]
- 142 **Carter AM**, Moayyedi P, Catto A, Heppell RM, Axon AT, Grant PJ. The influence of Helicobacter pylori status on circulating levels of the coagulation factors fibrinogen, von Willebrand factor, factor VII, and factor VIII. *Helicobacter* 1996; **1**: 65-69 [PMID: 9398916 DOI: 10.1111/j.1523-5378.1996.tb00011.x]
- 143 **Ikeda A**, Iso H, Sasazuki S, Inoue M, Tsugane S; JPHC Study Group. The combination of Helicobacter pylori- and cytotoxin-associated gene-A seropositivity in relation to the risk of myocardial infarction in middle-aged Japanese: The Japan Public Health Center-based study. *Atherosclerosis* 2013; **230**: 67-72 [PMID: 23958254 DOI: 10.1016/j.atherosclerosis.2013.06.013]
- 144 **Liu J**, Wang F, Shi S. Helicobacter pylori Infection Increase the Risk of Myocardial Infarction: A Meta-Analysis of 26 Studies Involving more than 20,000 Participants. *Helicobacter* 2015; **20**: 176-183 [PMID: 25382293 DOI: 10.1111/hel.12188]
- 145 **Hughes WS**. An hypothesis: the dramatic decline in heart attacks in the United States is temporally related to the decline in duodenal ulcer disease and Helicobacter pylori infection. *Helicobacter* 2014; **19**: 239-241 [PMID: 24689964 DOI: 10.1111/hel.12123]
- 146 **Oldenburg B**, Diepersloot RJ, Hoekstra JB. High seroprevalence of Helicobacter pylori in diabetes mellitus patients. *Dig Dis Sci* 1996; **41**: 458-461 [PMID: 8617115 DOI: 10.1007/BF02282318]
- 147 **Hsieh MC**, Wang SS, Hsieh YT, Kuo FC, Soon MS, Wu DC. Helicobacter pylori infection associated with high HbA1c and type 2 diabetes. *Eur J Clin Invest* 2013; **43**: 949-956 [PMID: 23879740 DOI: 10.1111/eci.12124]
- 148 **Bégué RE**, Gómez R, Compton T, Vargas A. Effect of Helicobacter pylori eradication in the glycemia of children with type 1 diabetes: a preliminary study. *South Med J* 2002; **95**: 842-845 [PMID: 12190218 DOI: 10.1097/00007611-200295080-00012]
- 149 **Demir M**, Gokturk HS, Ozturk NA, Kulaksizoglu M, Serin E, Yilmaz U. Helicobacter pylori prevalence in diabetes mellitus patients with dyspeptic symptoms and its relationship to glycemic control and late complications. *Dig Dis Sci* 2008; **53**: 2646-2649 [PMID: 18320319 DOI: 10.1007/s10620-007-0185-7]
- 150 **Yang GH**, Wu JS, Yang YC, Huang YH, Lu FH, Chang CJ. Gastric Helicobacter pylori infection associated with risk of diabetes mellitus, but not prediabetes. *J Gastroenterol Hepatol* 2014; **29**: 1794-1799 [PMID: 24731067 DOI: 10.1111/jgh.12617]
- 151 **Horikawa C**, Kodama S, Fujihara K, Hirasawa R, Yachi Y, Suzuki A, Hanyu O, Shimano H, Sone H. High risk of failing eradication of Helicobacter pylori in patients with diabetes: a meta-analysis. *Diabetes Res Clin Pract* 2014; **106**: 81-87 [PMID: 25110103 DOI: 10.1016/j.diabres.2014.07.009]
- 152 **Ibrahim A**, Zaher T, Ghonemy TA, El-Azim SA, El-Azim MA, Ramadan A. Impact of cytotoxin-associated gene A of Helicobacter pylori strains on microalbuminuria in type 2 diabetes. *Saudi J Kidney Dis Transpl* 2010; **21**: 694-700 [PMID: 20587874]
- 153 **Chen Y**, Blaser MJ. Association between gastric Helicobacter pylori colonization and glycated hemoglobin levels. *J Infect Dis* 2012; **205**: 1195-1202 [PMID: 22427676 DOI: 10.1093/infdis/jis106]
- 154 **Akanuma M**, Yanai A, Sakamoto K, Hirata Y, Yamaji Y, Kawazu S, Maeda S. Influence of Helicobacter pylori eradication on the management of type 2 diabetes. *Hepatogastroenterology* 2012; **59**: 641-645 [PMID: 22328266 DOI: 10.5754/hge11960]
- 155 **Wang X**, Bao W, Liu J, Ouyang YY, Wang D, Rong S, Xiao X, Shan ZL, Zhang Y, Yao P, Liu LG. Inflammatory markers and risk of type 2 diabetes: a systematic review and meta-analysis. *Diabetes Care* 2013; **36**: 166-175 [PMID: 23264288 DOI: 10.2337/dc12-0702]
- 156 **Jeon CY**, Haan MN, Cheng C, Clayton ER, Mayeda ER, Miller JW, Aiello AE. Helicobacter pylori infection is associated with an increased rate of diabetes. *Diabetes Care* 2012; **35**: 520-525 [PMID: 22279028 DOI: 10.2337/dc11-1043]
- 157 **Calle MC**, Fernandez ML. Inflammation and type 2 diabetes. *Diabetes Metab* 2012; **38**: 183-191 [PMID: 22252015 DOI: 10.1016/j.diabet.2011.11.006]
- 158 **Wang F**, Fu Y, Lv Z. Association of Helicobacter pylori infection with diabetic complications: a meta-analysis. *Endocr Res* 2014; **39**: 7-12 [PMID: 23879556 DOI: 10.3109/07435800.2013.794426]
- 159 **Yanik S**, Dogan Z, Sarikaya M, Ergul B, Filik L. Helicobacter pylori eradication reduces microalbuminuria in type-2 diabetic patients: a prospective study. *Acta Gastroenterol Belg* 2014; **77**: 235-239 [PMID: 25090822]
- 160 **Vafaeimanesh J**, Parham M, Seyyedmajidi M, Bagherzadeh M. Helicobacter pylori infection and insulin resistance in diabetic and nondiabetic population. *ScientificWorldJournal* 2014; **2014**: 391250 [PMID: 25405220 DOI: 10.1155/2014/391250]
- 161 **Chung GE**, Heo NJ, Park MJ, Chung SJ, Kang HY, Kang SJ. Helicobacter pylori seropositivity in diabetic patients is associated with microalbuminuria. *World J Gastroenterol* 2013; **19**: 97-102 [PMID: 23326169 DOI: 10.3748/wjg.v19.i1.97]
- 162 **Zhou X**, Liu W, Gu M, Zhou H, Zhang G. Helicobacter pylori infection causes hepatic insulin resistance by the c-Jun/miR-203/SOCS3 signaling pathway. *J Gastroenterol* 2015; **50**: 1027-1040 [PMID: 25689935 DOI: 10.1007/s00535-015-1051-6]
- 163 **Aydemir S**, Bayraktaroglu T, Sert M, Sokmen C, Atmaca H, Mungan G, Gun BD, Borazan A, Ustundag Y. The effect of Helicobacter pylori on insulin resistance. *Dig Dis Sci* 2005; **50**: 2090-2093 [PMID: 16240220 DOI: 10.1007/s10620-005-3012-z]
- 164 **Gunji T**, Matsuhashi N, Sato H, Fujibayashi K, Okumura M, Sasabe N, Urabe A. Helicobacter pylori infection significantly increases insulin resistance in the asymptomatic Japanese population. *Helicobacter* 2009; **14**: 144-150 [PMID: 19751440 DOI: 10.1111/j.1523-5378.2009.00705.x]
- 165 **Eshraghian A**, Hashemi SA, Hamidian Jahromi A, Eshraghian H, Masoompour SM, Davarpanah MA, Eshraghian K, Taghavi SA.

- Helicobacter pylori* infection as a risk factor for insulin resistance. *Dig Dis Sci* 2009; **54**: 1966-1970 [PMID: 19009348 DOI: 10.1007/s10620-008-0557-7]
- 166 **Naja F**, Nasreddine L, Hwalla N, Moghames P, Shoaib H, Fatfat M, Sibai A, Gali-Muhtasib H. Association of *H. pylori* infection with insulin resistance and metabolic syndrome among Lebanese adults. *Helicobacter* 2012; **17**: 444-451 [PMID: 23066847 DOI: 10.1111/j.1523-5378.2012.00970.x]
 - 167 **Chen TP**, Hung HF, Chen MK, Lai HH, Hsu WF, Huang KC, Yang KC. *Helicobacter Pylori* Infection is Positively Associated with Metabolic Syndrome in Taiwanese Adults: a Cross-Sectional Study. *Helicobacter* 2015; **20**: 184-191 [PMID: 25582223 DOI: 10.1111/hel.12190]
 - 168 **Niemelä S**, Karttunen T, Korhonen T, Läärä E, Karttunen R, Ikäheimo M, Kesäniemi YA. Could *Helicobacter pylori* infection increase the risk of coronary heart disease by modifying serum lipid concentrations? *Heart* 1996; **75**: 573-575 [PMID: 8697159 DOI: 10.1136/hrt.75.6.573]
 - 169 **Laurila A**, Bloigu A, Näyhä S, Hassi J, Leinonen M, Saikku P. Association of *Helicobacter pylori* infection with elevated serum lipids. *Atherosclerosis* 1999; **142**: 207-210 [PMID: 9920523 DOI: 10.1016/S0021-9150(98)00194-4]
 - 170 **Chen Y**, Blaser MJ. Inverse associations of *Helicobacter pylori* with asthma and allergy. *Arch Intern Med* 2007; **167**: 821-827 [PMID: 17452546 DOI: 10.1001/archinte.167.8.821]
 - 171 **Wang Q**, Yu C, Sun Y. The association between asthma and *Helicobacter pylori*: a meta-analysis. *Helicobacter* 2013; **18**: 41-53 [PMID: 23067334 DOI: 10.1111/hel.12012]
 - 172 **Zhou X**, Wu J, Zhang G. Association between *Helicobacter pylori* and asthma: a meta-analysis. *Eur J Gastroenterol Hepatol* 2013; **25**: 460-468 [PMID: 23242126 DOI: 10.1097/MEG.0b013e32835c280a]
 - 173 **Blaser MJ**, Chen Y, Reibman J. Does *Helicobacter pylori* protect against asthma and allergy? *Gut* 2008; **57**: 561-567 [PMID: 18194986 DOI: 10.1136/gut.2007.133462]
 - 174 **Amberbir A**, Medhin G, Erku W, Alem A, Simms R, Robinson K, Fogarty A, Britton J, Venn A, Davey G. Effects of *Helicobacter pylori*, geohelminth infection and selected commensal bacteria on the risk of allergic disease and sensitization in 3-year-old Ethiopian children. *Clin Exp Allergy* 2011; **41**: 1422-1430 [PMID: 21831135 DOI: 10.1111/j.1365-2222.2011.03831.x]
 - 175 **Oertli M**, Sundquist M, Hitzler I, Engler DB, Arnold IC, Reuter S, Maxeiner J, Hansson M, Taube C, Quiding-Järbrink M, Müller A. DC-derived IL-18 drives Treg differentiation, murine *Helicobacter pylori*-specific immune tolerance, and asthma protection. *J Clin Invest* 2012; **122**: 1082-1096 [PMID: 22307326 DOI: 10.1172/JCI61029]
 - 176 **Oertli M**, Noben M, Engler DB, Semper RP, Reuter S, Maxeiner J, Gerhard M, Taube C, Müller A. *Helicobacter pylori* γ -glutamyl transpeptidase and vacuolating cytotoxin promote gastric persistence and immune tolerance. *Proc Natl Acad Sci USA* 2013; **110**: 3047-3052 [PMID: 23382221 DOI: 10.1073/pnas.1211248110]
 - 177 **Memon AA**, Hussein NR, Miendje Deyi VY, Burette A, Atherton JC. Vacuolating cytotoxin genotypes are strong markers of gastric cancer and duodenal ulcer-associated *Helicobacter pylori* strains: a matched case-control study. *J Clin Microbiol* 2014; **52**: 2984-2989 [PMID: 24920772 DOI: 10.1128/JCM.00551-14]
 - 178 **Cook KW**, Letley DP, Ingram RJ, Staples E, Skjoldmose H, Atherton JC, Robinson K. CCL20/CCR6-mediated migration of regulatory T cells to the *Helicobacter pylori*-infected human gastric mucosa. *Gut* 2014; **63**: 1550-1559 [PMID: 24436142 DOI: 10.1136/gutjnl-2013-306253]
 - 179 **Engler DB**, Reuter S, van Wijck Y, Urban S, Kyburz A, Maxeiner J, Martin H, Yogeve N, Waisman A, Gerhard M, Cover TL, Taube C, Müller A. Effective treatment of allergic airway inflammation with *Helicobacter pylori* immunomodulators requires BATF3-dependent dendritic cells and IL-10. *Proc Natl Acad Sci USA* 2014; **111**: 11810-11815 [PMID: 25074917 DOI: 10.1073/pnas.1410579111]
 - 180 **Grad YH**, Lipsitch M, Aiello AE. Secular trends in *Helicobacter pylori* seroprevalence in adults in the United States: evidence for sustained race/ethnic disparities. *Am J Epidemiol* 2012; **175**: 54-59 [PMID: 22085628 DOI: 10.1093/aje/kwr288]
 - 181 **Sheikh A**, Strachan DP. The hygiene theory: fact or fiction? *Curr Opin Otolaryngol Head Neck Surg* 2004; **12**: 232-236 [PMID: 15167035 DOI: 10.1097/01.moo.0000122311.13359.30]
 - 182 **Molina-Infante J**, Gutierrez-Junquera C, Savarino E, Penagini R, Modolell I, Bartolo O, Prieto-García A, Mauro A, Alcedo J, Perelló A, Guarner-Argente C, Alcaide N, Vegas AM, Barros-García P, Murzi-Pulgar M, Perona M, Gisbert JP, Lucendo AJ; Upper GI Tract Study Group from the Spanish Gastroenterological Association (AEG). *Helicobacter pylori* infection does not protect against eosinophilic esophagitis: results from a large multicenter case-control study. *Am J Gastroenterol* 2018; [Epub ahead of print] [PMID: 29545632 DOI: 10.1038/s41395-018-0035-6]
 - 183 **Doğan Z**, Filik L, Ergül B, Sarikaya M, Akbal E. Association between *Helicobacter pylori* and liver-to-spleen ratio: a randomized-controlled single-blind study. *Eur J Gastroenterol Hepatol* 2013; **25**: 107-110 [PMID: 23013624 DOI: 10.1097/MEG.0b013e3283590c10]
 - 184 **Waluga M**, Kukla M, Żorniak M, Bacik A, Kotulski R. From the stomach to other organs: *Helicobacter pylori* and the liver. *World J Hepatol* 2015; **7**: 2136-2146 [PMID: 26328025 DOI: 10.4254/wjh.v7.i18.2136]
 - 185 **Polyzos SA**, Kountouras J, Zavos C, Deretzi G. The association between *Helicobacter pylori* infection and insulin resistance: a systematic review. *Helicobacter* 2011; **16**: 79-88 [PMID: 21435084 DOI: 10.1111/j.1523-5378.2011.00822.x]
 - 186 **Chen CX**, Mao YS, Foster P, Zhu ZW, Du J, Guo CY. Possible association between *Helicobacter pylori* infection and nonalcoholic fatty liver disease. *Appl Physiol Nutr Metab* 2017; **42**: 295-301 [PMID: 28177748 DOI: 10.1139/apnm-2016-0499]
 - 187 **Boutari C**, Perakakis N, Mantzoros CS. Association of Adipokines with Development and Progression of Nonalcoholic Fatty Liver Disease. *Endocrinol Metab* (Seoul) 2018; **33**: 33-43 [PMID: 29589386 DOI: 10.3803/EnM.2018.33.1.33]
 - 188 **Fukuda Y**, Bamba H, Okui M, Tamura K, Tanida N, Satomi M, Shimoyama T, Nishigami T. *Helicobacter pylori* infection increases mucosal permeability of the stomach and intestine. *Digestion* 2001; **63** Suppl 1: 93-96 [PMID: 11173917 DOI: 10.1159/000051918]
 - 189 **Sumida Y**, Kanemasa K, Imai S, Mori K, Tanaka S, Shimokobe H, Kitamura Y, Fukumoto K, Kakutani A, Ohno T, Taketani H, Seko Y, Ishiba H, Hara T, Okajima A, Yamaguchi K, Moriguchi M, Mitsuyoshi H, Yasui K, Minami M, Itoh Y. *Helicobacter pylori* infection might have a potential role in hepatocyte ballooning in nonalcoholic fatty liver disease. *J Gastroenterol* 2015; **50**: 996-1004 [PMID: 25622927 DOI: 10.1007/s00535-015-1039-2]
 - 190 **Basso D**, Plebani M, Kusters JG. Pathogenesis of *Helicobacter pylori* infection. *Helicobacter* 2010; **15** Suppl 1: 14-20 [PMID: 21054648 DOI: 10.1111/j.1523-5378.2010.00781.x]
 - 191 **Hotamisligil GS**, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF- α - and obesity-induced insulin resistance. *Science* 1996; **271**: 665-668 [PMID: 8571133]
 - 192 **Ohashi K**, Parker JL, Ouchi N, Higuchi A, Vita JA, Gokce N, Pedersen AA, Kalthoff C, Tullin S, Sams A, Summer R, Walsh K. Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. *J Biol Chem* 2010; **285**: 6153-6160 [PMID: 20028977 DOI: 10.1074/jbc.M109.088708]
 - 193 **Ki MR**, Goo MJ, Park JK, Hong IH, Ji AR, Han SY, You SY, Lee EM, Kim AY, Park SJ, Lee HJ, Kim SY, Jeong KS. *Helicobacter pylori* accelerates hepatic fibrosis by sensitizing transforming growth factor- β 1-induced inflammatory signaling. *Lab Invest* 2010; **90**: 1507-1516 [PMID: 20531291 DOI: 10.1038/labinvest.2010.109]
 - 194 **Lee A**, O'Rourke J, De Ungria MC, Robertson B, Daskalopoulos G, Dixon MF. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology* 1997; **112**: 1386-1397 [PMID: 9098027 DOI: 10.1016/S0016-5085(97)70155-0]
 - 195 **Goo MJ**, Ki MR, Lee HR, Yang HJ, Yuan DW, Hong IH, Park JK, Hong KS, Han JY, Hwang OK, Kim DH, Do SH, Cohn RD, Jeong

- KS. *Helicobacter pylori* promotes hepatic fibrosis in the animal model. *Lab Invest* 2009; **89**: 1291-1303 [PMID: 19736546 DOI: 10.1038/labinvest.2009.90]
- 196 **Smyk DS**, Koutsoumpas AL, Mytilinaiou MG, Rigopoulou EI, Sakkas LI, Bogdanos DP. *Helicobacter pylori* and autoimmune disease: cause or bystander. *World J Gastroenterol* 2014; **20**: 613-629 [PMID: 24574735 DOI: 10.3748/wjg.v20.i3.613]
 - 197 **Hasni S**, Ippolito A, Illei GG. *Helicobacter pylori* and autoimmune diseases. *Oral Dis* 2011; **17**: 621-627 [PMID: 21902767 DOI: 10.1111/j.1601-0825.2011.01796.x]
 - 198 **Goo MJ**, Ki MR, Lee HR, Hong IH, Park JK, Yang HJ, Yuan DW, Hwang OK, Do SH, Yoo SE, Jeong KS. Primary biliary cirrhosis, similar to that in human beings, in a male C57BL/6 mouse infected with *Helicobacter pylori*. *Eur J Gastroenterol Hepatol* 2008; **20**: 1045-1048 [PMID: 18787477 DOI: 10.1097/MEG.0b013e3282f5e9db]
 - 199 **Shapira Y**, Agmon-Levin N, Renaudineau Y, Porat-Katz BS, Barzilai O, Ram M, Youinou P, Shoenfeld Y. Serum markers of infections in patients with primary biliary cirrhosis: evidence of infection burden. *Exp Mol Pathol* 2012; **93**: 386-390 [PMID: 23022373 DOI: 10.1016/j.yexmp.2012.09.012]
 - 200 **Nilsson HO**, Taneera J, Castedal M, Glatz E, Olsson R, Wadström T. Identification of *Helicobacter pylori* and other *Helicobacter* species by PCR, hybridization, and partial DNA sequencing in human liver samples from patients with primary sclerosing cholangitis or primary biliary cirrhosis. *J Clin Microbiol* 2000; **38**: 1072-1076 [PMID: 10698999]
 - 201 **Bogdanos DP**, Baum H, Gunsar F, Arioli D, Polymeros D, Ma Y, Burroughs AK, Vergani D. Extensive homology between the major immunodominant mitochondrial antigen in primary biliary cirrhosis and *Helicobacter pylori* does not lead to immunological cross-reactivity. *Scand J Gastroenterol* 2004; **39**: 981-987 [PMID: 15513338 DOI: 10.1080/00365520410003236]
 - 202 **Krasinskas AM**, Yao Y, Randhawa P, Dore MP, Sepulveda AR. *Helicobacter pylori* may play a contributory role in the pathogenesis of primary sclerosing cholangitis. *Dig Dis Sci* 2007; **52**: 2265-2270 [PMID: 17393314 DOI: 10.1007/s10620-007-9803-7]
 - 203 **Casswall TH**, Németh A, Nilsson I, Wadström T, Nilsson HO. *Helicobacter* species DNA in liver and gastric tissues in children and adolescents with chronic liver disease. *Scand J Gastroenterol* 2010; **45**: 160-167 [PMID: 20095882 DOI: 10.3109/00365520903426915]
 - 204 **Rabelo-Gonçalves EM**, Roesler BM, Zeitune JM. Extragastric manifestations of *Helicobacter pylori* infection: Possible role of bacterium in liver and pancreas diseases. *World J Hepatol* 2015; **7**: 2968-2979 [PMID: 26730276 DOI: 10.4254/wjh.v7.i30.2968]
 - 205 **Tu QV**, Okoli AS, Kovach Z, Mendz GL. Hepatocellular carcinoma: prevalence and molecular pathogenesis of *Helicobacter* spp. *Future Microbiol* 2009; **4**: 1283-1301 [PMID: 19995189 DOI: 10.2217/fmb.09.90]
 - 206 **Avenaud P**, Marais A, Monteiro L, Le Bail B, Bioulac Sage P, Balabaud C, Mégraud F. Detection of *Helicobacter* species in the liver of patients with and without primary liver carcinoma. *Cancer* 2000; **89**: 1431-1439 [PMID: 11013355 DOI: 10.1002/1097-0142(20001001)89:73.0.CO;2-5]
 - 207 **Nilsson HO**, Mulchandani R, Tranberg KG, Stenram U, Wadström T. *Helicobacter* species identified in liver from patients with cholangiocarcinoma and hepatocellular carcinoma. *Gastroenterology* 2001; **120**: 323-324 [PMID: 11246512 DOI: 10.1053/gast.2001.21382]
 - 208 **Dore MP**, Realdi G, Mura D, Graham DY, Sepulveda AR. *Helicobacter* infection in patients with HCV-related chronic hepatitis, cirrhosis, and hepatocellular carcinoma. *Dig Dis Sci* 2002; **47**: 1638-1643 [PMID: 12141829 DOI: 10.1023/A:1015848009444]
 - 209 **Verhoef C**, Pot RG, de Man RA, Zondervan PE, Kuipers EJ, IJzermans JN, Kusters JG. Detection of identical *Helicobacter* DNA in the stomach and in the non-cirrhotic liver of patients with hepatocellular carcinoma. *Eur J Gastroenterol Hepatol* 2003; **15**: 1171-1174 [PMID: 14560149 DOI: 10.1097/00042737-200311000-00004]
 - 210 **Pellicano R**, Mazzaferro V, Grigioni WF, Cutufia MA, Fagoonee S, Silengo L, Rizzetto M, Ponzetto A. *Helicobacter* species sequences in liver samples from patients with and without hepatocellular carcinoma. *World J Gastroenterol* 2004; **10**: 598-601 [PMID: 14966925 DOI: 10.3748/wjg.v10.i4.598]
 - 211 **Esmat G**, El-Bendary M, Zakarya S, Ela MA, Zalata K. Role of *Helicobacter pylori* in patients with HCV-related chronic hepatitis and cirrhosis with or without hepatocellular carcinoma: possible association with disease progression. *J Viral Hepat* 2012; **19**: 473-479 [PMID: 22676359 DOI: 10.1111/j.1365-2893.2011.01567.x]

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ATP-binding cassette transporters in progression and clinical outcome of pancreatic cancer: What is the way forward?

Aleksandra Adamska, Marco Falasca

Aleksandra Adamska, Marco Falasca, Metabolic Signalling Group, School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth 6102, WA, Australia

ORCID number: Aleksandra Adamska (0000-0002-7152-4149); Marco Falasca (0000-0002-9801-7235).

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Correspondence to: Marco Falasca, PhD, Professor, Metabolic Signalling Group, School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, GPO Box U1987, Perth 6102, WA, Australia. marco.falasca@curtin.edu.au
Telephone: +61-8-92669712

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive diseases and is characterized by high chemoresistance, leading to the lack of effective therapeutic approaches and grim prognosis. Despite increasing understanding of the mechanisms of chemoresistance in cancer and the role of ATP-binding cassette (ABC) transporters in this resistance, the therapeutic potential of their pharmacological inhibition has not been successfully exploited yet. In spite of the discovery of potent pharmacological modulators of ABC transporters, the results obtained in clinical trials have been so far disappointing, with high toxicity levels impairing their successful administration to the patients. Critically, although ABC transporters have been mostly studied for their involvement in development of multidrug resistance (MDR), in recent years the contribution of ABC transporters to cancer initiation and progression has emerged as an important area of research, the understanding of which could significantly influence the development of more specific and efficient therapies. In this review, we explore the role of ABC transporters in the development and progression of malignancies, with focus on PDAC. Their established involvement in development of MDR will be also presented. Moreover, an emerging role for ABC transporters as prognostic tools for patients' survival will be discussed, demonstrating the therapeutic potential of ABC transporters in cancer therapy.

Key words: Pancreatic ductal adenocarcinoma; Multi-drug resistance; ATP-binding cassette transporters; Targeted therapies; Pancreatic ductal adenocarcinoma prognosis; Predictive markers

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Core tip: Pancreatic cancer is one of the deadliest cancers due to its highly aggressive biology and resistance to broad range of therapeutics. Expression of ATP-binding cassette (ABC) transporters by cancer cells is one of the main mechanisms responsible for the lowered drug accumulation. However, the attempts made in multidrug resistance reversal by the inhibition of their activity have not provided satisfactory results in clinical trials. Nevertheless, current knowledge on the role played by ABC transporters in carcinogenesis beyond chemoresistance, could create the opportunity for the development of novel, direct targeted therapeutic strategies. Additionally, the association between ABC transporters expression and pancreatic ductal adenocarcinoma patients' prognosis and response to applied therapies confirms their pharmacological potential.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most fatal diseases in western world. Although not one of the leading causes of death, PDAC is certainly to be considered amid the most unfavourable cancers, ranking at 4th place in terms of death rate, with a 7%-8% chance of 5-year survival in United States^[1]. Despite the progress made in understanding the biology and in the treatment of different cancer types, the mortality of PDAC patients still nearly equals its incidence and has not changed remarkably for the last few decades. The dismal prognosis of PDAC is the result of multiple factors including an aggressive nature, chemo- and radio-resistance and the lack of effective treatments and diagnostic tools. Therefore, when diagnosed, the vast majority of PDAC patients present with metastatic disease, not susceptible for surgery^[2]. Only one fifth of the patients have the tumour resected and, unfortunately, most of them eventually relapse. Post-operative chemo- and radiotherapy are usually applied in order to delay tumour recurrence; nevertheless, high resistance and the heterogeneous nature of pancreatic tumours impede its treatment^[3].

Pancreatic cancer pathology is a multistep process. It arises as an accumulation of abnormalities, both genetic and physiological, progressing through 3 stages of precursor lesions called pancreatic intraepithelial neoplasias (PanINs) before transforming into a fully differentiated tumour^[4]. The substantial number of genetic modifications and consequent dysregulation of the wide range of essential signalling pathways

accompanying these processes make PDAC highly heterogeneous^[5]. Also, the variability of mutations between patients as well as within the same tumour contributes to its high resistance to applied therapy. High heterogeneity of PDAC is expressed also phenotypically. Genetically diverse subclones, possessing different metabolic and functional characteristics, exist within a tumour. Recent evidence shows that one of the populations acquires characteristics similar to stem cells, which enables it to survive during stressful conditions and is partly responsible for cancer relapse after treatment^[6]. Furthermore, PDAC cell plasticity, which plays a role in epithelial to mesenchymal transition (EMT), facilitates metastatic spread and adds to the dismal prognosis^[7]. Moreover, one of the main characteristics of PDAC, responsible for therapies' failure, is the formation of dense desmoplastic reaction, influencing cancer progression and impeding drug delivery to the tumour^[8]. The interplay between tumour cells and stromal components (pancreatic stellate cells (PSCs), immune cells, cytokines or extracellular matrix proteins) influences cell metabolism, drug delivery and distribution. In addition, the existence of a rich tumour microenvironment (TME), influencing cancer cell functions and favouring chemoresistance, has been recently claimed to be an essential factor in cancer stem cell initiation and promotion^[9].

PDAC RESISTANCE TO THERAPIES

On account of PDAC aggressive nature and its resistance to therapies, no successful treatment has been introduced so far^[10]. In fact, until recently the gold standard in PDAC treatment was gemcitabine. Applied as a first line therapy drug since 1997, gemcitabine modestly improved patients' perspectives, increasing overall survival (OS) for 6 mo compared to previously used fluorouracil (5-FU)^[11]. Since that time, attempts have been made to increase the efficacy of PDAC treatment and prolong patient survival; however, only modest or statistically insignificant improvements have been achieved so far. In the last years, two new drug regimens, ABRAXANE and FOLFIRINOX have been introduced^[12,13]. However, their application did not increase OS to a meaningful degree when compared to gemcitabine, at the same time escalating the frequency of adverse events. Nevertheless, both treatments have obtained FDA approval and currently ABRAXANE combined with gemcitabine is acknowledged as a standard first-line therapy for pancreatic cancer. Considering the high number of genes altered during PDAC progression, targeted therapies emerged as a potential therapeutic tool. Many small inhibitors have been developed as single agents or applied in combination with gemcitabine or ABRAXANE to enhance their efficacy^[14-18]. However, the vast majority of them failed to improve patients' survival in the clinical settings. Therefore, it remains pivotal to gain better knowledge on the mechanisms of PDAC chemoresistance and to

find novel therapeutic strategies in order to develop more effective treatment regimens.

Among other factors, the failure of PDAC treatment has been attributed to local recurrence and liver metastasis and importantly, to its high chemoresistance, both intrinsic and acquired. The phenomenon called multi-drug resistance (MDR), which is characterized by resistance to a broad spectrum of structurally diversified compounds, has been confirmed as one of the main reasons for the inefficiency of PDAC therapies, leading to tragic health and economic consequences.

There are multiple factors contributing to the development of MDR in pancreatic cancer, such as decreased drug uptake, accelerated drug metabolism and DNA repair, blocking of apoptotic pathways, metabolic changes and the presence of highly resistant stem-like cells. Also, high heterogeneity of the tumour, dense stroma and hypoxia impairing drug delivery and constitutive activation of several signalling pathways, including K-Ras, PI3K/Akt, Notch or NF- κ B, with the latter being additionally enhanced during chemo- and radiotherapy, all confer the modest response of PDAC to applied therapies^[19-23]. Moreover EMT, frequently observed in PDAC tumours, has been implicated in conferring its resistance. Also, acquired mutations in targeted genes and reactivation of parallel pathways add to the therapy failing. However, in most cases the interplay between several of these processes is essential for chemoresistance development^[24]. Additionally, high expression of transmembrane proteins belonging to the ATP-binding cassette (ABC) transporter family in tumour specimens is one of the major factors contributing to increased drug efflux and has been connected with MDR, adding to the poor response of PDAC to treatments^[25-28]. Apart from drug extrusion, as integral membrane constituents, ABC transporters normally regulate the distribution of a wide variety of molecules, influencing different pathways and biological processes, which suggests their more direct impact on cell physiology and possibly, carcinogenesis. Therefore, the understanding of the role of ABC transporters both in healthy physiology and in cancer is crucial for the development of specific, potent and safe inhibitors that might be used in PDAC therapy.

ABC TRANSPORTERS AS MULTI-DRUG RESISTANCE MECHANISM

One of the main obstacles in cancer therapy is the resistance, both constitutive and acquired to administered drugs. As aforementioned, one of the processes responsible for drug resistance is the decreased intracellular accumulation of the drugs caused by their efflux from the cells induced by the expression of membrane drug transporters belonging to the ABC family.

The family of ABC transporters is a highly conserved family of proteins, expressed in all organisms, which

implies their relevance in many biological functions. To date, 48 human genes and one pseudogene encoding the members of ABC family have been described and grouped into 7 subfamilies (ABCA-G), based on their sequence and structural similarity^[29,30]. ABC transporters are integral transmembrane proteins which, by utilizing energy obtained from ATP hydrolysis, which drives the progressive conformational changes in their domains, shuffle molecules across the plasma and intracellular membranes against their gradient^[31,32] (Figure 1). The structure of ABC transporters is highly conserved and consists of two hydrophobic transmembrane domains (TMDs), which form a pore in the membrane creating substrate-binding environment linked to two hydrophilic nucleotide-binding domains (NBDs) localized in the cytosol^[33,34]. ABC transporters are reported to export a wide variety of structurally diverse endogenous ligands including amino acids, peptides, vitamins, sugars, hormones, ions, lipids and xenobiotics^[26,32,35-37]. For example, ABCB1 has been reported to be able to transport more than 200 structurally diversified molecules^[38-41]. Additionally, ABC transporters are known to excrete toxins from kidneys, gastrointestinal tract and liver, demonstrating a protective role in those tissues^[42]. Few ABC transporters, *e.g.*, ABCC7- cystic fibrosis transmembrane conductance regulator (CFTR) or ABCC8- the sulphonyl urea receptor (SUR1), are not directly involved in transport of molecules across the membrane but use the ATP hydrolysis to regulate the activity of Cl⁻ and K⁺ channels respectively^[43]. In healthy physiology, ABC transporters are expressed in a wide variety of tissues, mainly associated with biological barriers (Table 1). As an example, ABCC1 is expressed in kidneys, intestine, ovaries, adrenal glands, colon, stomach, testes, lungs and blood-brain barrier and ABCB1 is mostly expressed in gastrointestinal tract, pancreas, kidneys, brain and adrenal glands, where they are involved in diverse physiological functions and in excreting toxins from the cells^[40,44,45]. However, their enhanced levels have been found in different cancer types, suggesting the relevance of ABC transporters in cancer and its chemoresistance. So far, 15 of the transporters have been attributed the role of drug pumps, contributing to MDR *in vitro*^[46]. Especially, P glycoprotein (P-gp)/ABCB1, breast cancer resistance protein (BCRP)/ABCG2, multidrug resistance protein 1 (MRP1)/ABCC1 and other members of ABCC subfamily (*e.g.*, ABCC2, ABCC3) have been reported to be responsible for PDAC chemoresistance^[47].

Up to date, most research has been focused on P-gp, a member of the ABCB subfamily of transporters^[48,49]. It exports a wide variety of molecules of "amphipathic nature" including anthracyclines, HIV-protease inhibitors, calcium channel blockers, steroid hormones, antibiotics, lipids, taxanes and alkaloids^[50,51]. P-gp overexpression has been observed in several cancers including ovarian, colon, kidney or adrenocortical cancer, correlating with poor prognosis^[52,53]. Additionally,

Table 1 Selected ATP-binding cassette transporters, their normal physiological expression and overexpression in cancer tissues^[117,171]

ABC transporter	Tissue expression	Cancer overexpression	Correlation with PDAC survival (5-yr survival)
ABCA			
ABCA1	Lung, colon, liver, brain, testicles	Glioma, lung, testis, liver, colorectal, breast, renal cancer,	H: 21% L: 29%
ABCA7	Bone marrow, brain, kidney, colon, lung pancreas	Melanoma, Lung, cervical, stomach, endometrial, colorectal, pancreatic cancer	H: 38% L: 0%
ABCB			
ABCB1	Brain, blood-brain barrier, colon, liver, kidney, testis, placenta, small intestine, pancreas	Ovarian, breast, colon, kidney, adrenocortical cancer, AML	H: 34% L: 20%
ABCB4	Liver	Liver, lung, renal cancer, melanoma	H: 49% L: 22%
ABCC			
ABCC1	Kidney, colon, pancreas, lymph nodes, liver, testis, brain, blood-brain barrier, breasts, spleen,	Breast, lung, ovarian or prostate cancer, neuroblastoma	H: 13% L: 43%
ABCC2	Brain, lymph nodes, liver, colon, kidney, lung, testis, breasts, pancreas	Colorectal, liver, lung, gastric cancer	H: 29% L: 27%
ABCC3	Pancreas, liver, lymph nodes, lung, adrenal glands, colon, testis, spleen, small intestine	Pancreatic, liver, lung, colorectal, stomach, renal, breast cancer	H: 13% L: 41%
ABCC4	Brain, testis, colon, kidney adrenal glands, pancreas, liver, ovary, lung, spleen, breasts, skin, heart	Prostate, renal, lung, breast, ovarian, stomach cancer	H: 32% L: 23%
ABCC5	Lymph nodes, pancreas, kidney, testis, brain, colon, liver, heart, muscles	Lung, urothelial, breast, cervical, renal cancer, glioma	H: 34% L: 0%
ABCG			
ABCG1	Pancreas, liver, colon, kidney, brain, lung, lymph nodes, testis	Lung, renal, breast, endometrial, prostate, colorectal, cervical, pancreatic cancer, glioma	H: 34% L: 0%
ABCG2	Intestine, testis, colon, placenta, liver, kidney, small intestine	Liver, testis, prostate, renal cancer, glioma	H: 32% L: 23%
ABCG4	Brain, endocrine, testis, colon, liver, kidney	Glioma, melanoma, thyroid, head and neck, renal, testis, ovarian, endometrial cancer	H: 43% L: 23%

The correlation between the overexpression of the transporters in PDAC and observed 5-year survival is also demonstrated^[117]. H: High expression of the transporter; L: Low expression of the transporter. Statistically significant association is highlighted in bold. AML: Acute myeloid leukaemia; PDAC: Pancreatic ductal adenocarcinoma; ABC: ATP-binding cassette.

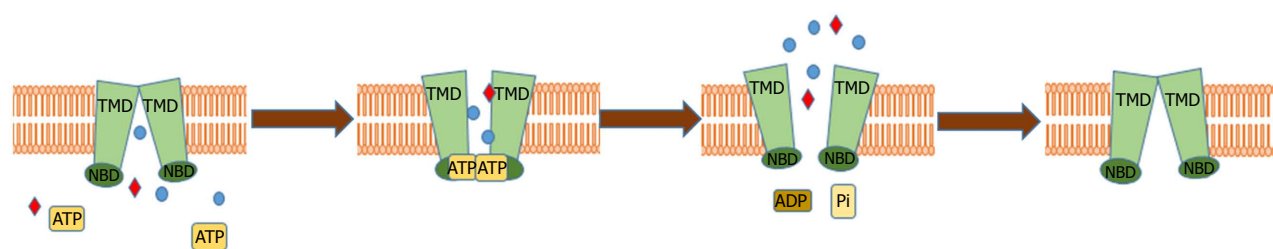


Figure 1 The schematic presentation of the mechanism of ATP-mediated ABC transporter substrate translocation. TMD: Transmembrane domain.

treatment-induced increase in ABCB1 expression has been noted in acute myeloid leukaemia (AML)^[54], breast and high-grade bladder cancer^[55]. ABCB1 is known to be responsible for developing drug resistance to neutral and cationic hydrophobic compounds, *e.g.*, to anthracyclines (daunorubicin, doxorubicin), colchicines, taxanes (paclitaxel, docetaxel), vinca alkaloids (*e.g.*, vincristine, vinblastine) and tyrosine kinase inhibitors (imatinib)^[56-58].

The main role in xenobiotic transport and drug resistance in many cancers has been attributed to the ABCC subfamily of transmembrane transporters^[59], with 9 out of 12 members being involved in MDR^[47,59]. The most studied of MDR proteins, ABCC1 (MPR1)

has been demonstrated to be expressed in several cancers, including breast, lung, ovarian and prostate cancer, showing the correlation between the expression of ABCC1 and poor patients' outcome^[60]. It has been suggested that ABCC1 expression may confer resistance to methotrexate, vinca alkaloids, anthracyclines and camptothecins^[47,61,62], influencing drug resistance in plethora of cancers. Additionally, cyclic nucleotides and their analogues (*e.g.*, gemcitabine) may be transported by ABCC4 and ABCC5^[62-64], potentially contributing to their ineffectiveness in PDAC therapy.

Resistance to doxorubicin, mitoxantrone, anthracyclines and topotecan (quinolone topoisomerase inhibitor)^[65] has been attributed to ABCG2 transporter^[66,67], which

functions mainly in the ovaries, brain, liver, prostate, placenta and small intestine^[68]. Additionally, increased ABCG2 expression has been reported in pluripotent stem cells, suggesting its role in the maintenance and protection of stem cells^[69].

Regardless of the remarkable increase in the knowledge on the ABC transporters structure and MDR induction achieved in the past few decades, the actual function and significance of these proteins is highly underexplored. It is known that in healthy physiology, ABC transporters are involved in drug absorption, distribution and elimination, determining bioavailability of administered drugs. Both apical and basolateral membranes of gastrointestinal tract and biological barriers, in which ABC transporter expression has been demonstrated, need to be penetrated by the drug to reach its target. Therefore, ABC transporters expression may influence pharmacokinetic characteristics of administered chemotherapeutics. Additionally, various other physiological roles have been assigned to ABC transporters such as export of fatty acids, cholesterol, peptides, sterols and xenobiotics. Many ABC transporters are involved in secretion of bioactive molecules and in the transport of signalling lipids, which contribution to cancer progression has been well established. As an example, ABCA1 is involved in reverse cholesterol transport as well as phospholipids transport to plasma membrane^[70,71]. Interestingly, recent studies demonstrated ABCC1 as an active player in progression of ovarian and prostate cancer^[72,73], by extrusion of lipids (lysophosphatidylinositol, sphingosine 1-phosphate) that have been previously attributed a crucial role in carcinogenesis^[73,74]. The changes in cancer cell proliferation, migration, invasion and resistance to apoptosis mediated by the activity of ABC transporters have been also widely documented^[75]. Considering that information, attention has been brought to the pivotal role played by ABC transporters in carcinogenesis beyond chemoresistance and to the correlation between their expression with cancer progression and aggressiveness. Nevertheless, this area is still overlooked and more studies need to be focused on this aspect of ABC transporters' activity in order to fully elucidate their role in cancer.

ABC TRANSPORTERS- DRIVERS OF PDAC PROGRESSION?

There have been very limited studies on the role and expression of ABC transporters in pancreatic cancer; however, strong correlation between few of their members and PDAC has been recently suggested. On the basis of mRNA analysis, the expression of ABCC1, ABCC3, ABCC4, ABCC5 and ABCG2 in both pancreatic cancer samples and in healthy pancreas has been demonstrated^[76,77] and was correlated with cell resistance to commonly applied chemotherapeutics^[78]. At the same time, ABCC6, ABCC8 and ABCC9 could not

be detected in any of the studied pancreatic cancer cell lines^[79]. Furthermore, more in depth analysis showed that although ABCG2, ABCC1 and ABCC4 levels did not differ significantly between tumour and healthy tissues, ABCC3 and ABCC5 were found to be remarkably overexpressed in PDAC specimens. Moreover, although expression of none of them could be coupled with cancer stage, the differentiation status and tumour grading were related with increased ABCC3 levels and correlated with poor survival, whereas no such correlation could be found for ABCC5.

ABCC3 transporter is involved in transporting of bile salts and organic ions^[80,81]. It has been also implicated in mediation of drug resistance, *e.g.*, to vincristine, methotrexate or etoposide; compounds used in clinical studies for PDAC treatments, which demonstrated only marginal effects^[82]. Moreover, its expression levels have been correlated with survival of patients after resection, suggesting possible predictive aspect of ABCC3 expression in PDAC.

ABCC5 is involved in transport of nucleotide analogues; therefore, it is tempting to speculate its involvement in excessive efflux of nucleotide analogues-based drugs, such as 5-FU or gemcitabine. In fact, although still controversial, it has been shown that ABCC5 is responsible for gemcitabine resistance in pancreatic cancer^[64,79,83]. Analysis of PDAC specimens demonstrated overexpression of ABCC5 transporter in samples resistant to gemcitabine, suggesting its involvement in the decreased efficiency of the drug. Furthermore, exposure of different PDAC cell lines to gemcitabine, as well as 5-FU/gemcitabine combination significantly increased the expression of ABCC5 demonstrating drug induced mechanism of PDAC cell resistance to the treatment^[79,84]. Therefore, although not directly associated with PDAC progression, the importance of ABCC5 in PDAC chemoresistance, both inherent and acquired, makes it a valuable drug target for the enhancement of the efficacy of applied therapies.

While the role of ABC transporters in mediating chemoresistance is well established, little is known about their direct, drug-efflux independent contribution to pancreatic cancer progression. Nevertheless, intensive studies in recent years suggest that beyond their role in drug resistance, the biological functions of ABC transporters are more complex. It has been proposed that tumour-promoting functions of ABC transporters are based on their ability to export active signalling molecules and hormones, which by autocrine or paracrine regulation activate cancer cells as well as tumour environment. Increasing interest in this area has demonstrated the significant impact of these proteins on invasion, migration and differentiation of malignant cells^[75]. Also, changes in metabolism as well as redox status, characteristics pivotal in PDAC tumorigenesis, may be induced by ABC transporters-released molecules.

One of the major events in PDAC development

is the metabolic switch, which occurs in response to decreased nutrient and oxygen supply^[85-87]. Increased glucose dependence and use of aerobic glycolysis for energy production, known as Warburg effect, allows quickly proliferating PDAC cells to survive under harsh conditions and is considered as one of the hallmarks of cancer^[88]. Additionally, glutamine dependence and increased protein breakdown add to cancer cell high proliferative abilities. However, a small population of cells with stem-like characteristics, which reside in the areas of the tumour lacking oxygen and glucose supply, are known to rely on mitochondrial oxidative phosphorylation rather than glycolysis, which results in increased ATP production. This phenomenon may add to increased activity of ABC transporters observed in cancer cells. Therefore, low oxygen and nutrient supply may contribute to PDAC resistance by increase of the ABC transporters levels and their ATP-dependent substrate transport, suggesting a possible mechanism of hypoxia-induced chemoresistance, tumour maintenance and cancer progression.

Apart from glucose and glutamine addiction, increased lipid metabolism and demand has been recently demonstrated for PDAC^[89,90]. Bioactive phospholipids are directly involved in the induction of cancer cell proliferation and thereby, cancer progression^[91]. Increase in the levels of saturated lipids helps cancer cells to acquire additional resistance to oxidative stress by consolidating the membranes. Both, *de novo* lipid synthesis and their increased uptake have been reported in PDAC^[92,93]. Moreover, enzymes involved in lipolysis and lipogenesis are overexpressed in PDAC and are usually correlated with poor prognosis^[90]. It has been demonstrated by our work that, in prostate and ovarian cancer, ABCC1-transported lysophosphatidylinositol activates GPR55 receptor forming an autocrine loop, which activation triggers signalling cascade inducing cell proliferation^[72]. Phospholipids transport has been also reported for another member of ABC transporter family, ABCG1. Therefore, it is tempting to suspect the existence of a similar mechanism, involving ABC transporter-mediated phospholipid activation of cancer cells in PDAC. An essential factor in PDAC cell survival is also cholesterol availability. As a component of lipid rafts, it influences membrane composition and integrity and interacts with membrane-bound proteins, facilitating activation of phosphorylation cascades^[90]. The essential role played by cholesterol in PDAC tumorigenesis limits the growth and division of PDAC cells, depending on its availability^[94]. A recent study by Mohelnikova-Duchonova *et al.*^[95] showed an upregulation in transcript levels of several ABC transporters in PDAC compared to non-neoplastic tissues. Particularly, upregulation of 2 members of ABCA family, ABCA1 and ABCA7 involved in cholesterol export, together with expression of ABCG1 transporting phosphatidylserine, phosphatidylcholine and sphingomyelin, suggests their involvement in cellular cholesterol imbalance in the disease^[95]. Another

of the characteristics of PDAC is the highly inflammatory environment, which actively promotes cancer cell proliferation and survival, angiogenesis and assists the metastatic spread^[96]. Chronic inflammation, that aids the tumorigenesis and at the same time is one of the main factors contributing to its initiation, is mediated by prostaglandin-mediated pathways. Therefore, the main inflammatory molecules- prostaglandins and leukotrienes are considered as significant players in PDAC development. The prostaglandin-mediated PDAC progression may involve activation of PI3K-Akt signalling pathway, a major player in PDAC progression, increase in VEGFA expression and stimulation of angiogenesis and support of the inflammatory environment^[97]. It is now known that several ABC transporters, mainly belonging to the ABCC subfamily (ABCC1, ABCC2, ABCC4) are involved in prostaglandins efflux outside of the cells, enabling the activation of the G protein-coupled receptors, triggering cancer progression^[75,98]. Therefore, the manipulation of ABC transporter activity blocking prostaglandin signalling represents an additional potential therapeutic tool. Additionally, due to the proved contribution of leukotriene C4 (LTC4) to PDAC progression^[99], its induced pathways have been widely studied as potential drug targets. Regarding the involvement of ABC transporters in leukotriene release, their inhibition presents an additional possibility for LTC4-signalling blockade, influencing cancer development.

Elevated levels of reactive oxygen species (ROS), inducing oxidative stress are also implicated in PDAC initiation and progression^[100]. One of the molecules responsible for the maintenance of redox status in homeostasis is glutathione (GSH)^[101], which transport is activated in response to oxidative stress. It is also involved in several signalling processes regulating cell proliferation, apoptosis or immune response. Several members of ABCC family (ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC7) and ABCG2 mediate glutathione transport, suggesting their involvement in cellular response to the oxidative stress. Also, ABCB10 has been implicated in cellular protection from oxidative stress^[102]. Moreover, oxidative stress induces the activation of NF- κ B and Nrf2 signalling, which in turn enhances expression of ABCB1, ABCG2 and ABCC2, additionally contributing to cancer cell resistance^[103,104]. Therefore, manipulation of the activity of ABC transporters in cancer cells might potentially increase their antioxidant capacity, which has been shown to provide additional anti-tumorigenic protection^[105].

Additionally, tumour environment and its engagement in cancer progression and metastatic spread has emerged as key player in carcinogenesis. Considering the significant role of stroma in PDAC progression and in the development of tumour chemoresistance, targeting its components presents a tempting approach in the development of novel therapies. However, the attempts to deplete stroma have not provided satisfactory results so far. The most promising combination of gemcitabine

and Hedgehog inhibitor IPI-926-03 tested by Olive *et al.*^[106] has failed due to high toxicity and lack of effectiveness in clinical trials^[107]. Currently, molecules targeting hyaluronic acid, combined with chemotherapy, are being tested in phase II and III clinical trials^[108]. Nevertheless, investigation of new approaches to target stroma in order to increase chemotherapy efficiency, as well as restraining tumour expansion remains essential. Recently, expression of several of ABC transporters in PDAC stroma has been reported. One of the main stromal components- macrophages- have been demonstrated to express several of the drug transporters, inter alia ABCC1 and ABCC3, contributing to both chemoresistance and tumour progression^[109]. Therefore, considering the involvement of ABC transporters in chemoresistance and an emerging role in tumorigenesis, therapies targeting ABC transporters might prove to be useful in depleting or reprogramming cancer stroma and reversing cancer resistance to applied drugs. Additionally, expression of few of ABC transporters in non-neoplastic tissues has been recently reported to influence PDAC progression and to be predictive of patients' overall response.

Finally, the most aggressive tumours are composed of non-differentiated cells possessing highly proliferative abilities^[75], called cancer stem cells (CSCs). In particular, the existence and high importance of CSCs in cancer resistance to chemotherapy and its involvement in disease recurrence has been suggested for PDAC. Interestingly, high expression of ABC transporters has been reported in less differentiated tumour zones, conferring them a more aggressive phenotype^[110-112], also in PDAC^[76]. Therefore recently, the interest in CSCs as drivers of resistance and aggressive nature has emerged in PDAC^[113,114]. A noticeable characteristic of cancer stem cells is the high expression of members of the ABC transporters family compared to more differentiated cells^[115]. Also, it is speculated that their expression profile may be considered as the indicator of stem cell formation and carcinogenic potential of the tissue^[116]. Considering the association of cell differentiation levels with its proliferative potential, the overexpression of ABC transporters in cancer stem cells highly supports their contribution to the more aggressive nature of the PDAC. Overexpression of ABC transporters in cancer stem cells may assist in their survival by efflux of xenobiotics, exhibiting protective roles, sustaining their proper performance and maintaining self-renewal characteristics. Additionally, their enhanced expression and activity in cancer cells and especially in CSCs, suggests an additional role in maintaining cancer cells aggressive biology and makes them an attractive therapeutical target.

ABC TRANSPORTERS EXPRESSION PROFILES AS PROGNOSTIC MARKERS IN PDAC

Although the investigation on the role of ABC transporters

in PDAC is still in its outset, the initial analysis suggests their probable contribution to PDAC development and points at potential beneficial clinical consequences. Database analysis showed that the high importance and the potential of ABC transporters as pharmacological targets in PDAC is reflected in the association of the expression of its individual members with the prognosis of patients' survival^[117]. Notable correlation between observed 5-year survival and expression of a majority of ABC transporters has been observed (Table 1); however, this discovered association is not uniform. Significant reduction in survival probability has been attributed to high expression of *e.g.*, ABCA1, ABCA12, ABCB1, ABCC1, ABCC3 or ABCC7. Expression of few other ABC transporters showed similar trend, nonetheless, their relationship with the OS was not remarkably pronounced. On the other hand, higher expression of a substantial number of ABC transporter genes has been correlated with increased chance of PDAC patients' survival. Among others, the expression of ABCA2, ABCA7, ABCB6 ABCB8, ABCC5 or AGCG1 in PDAC tissues most markedly correlated with prolonged 5-year survival, suggesting their-mediated release of molecules of anti-tumorigenic characteristics and favourable prognostic potential.

Considering the elevated expression of multiple ABC transporters in a vast majority of cancers and their redundancy in substrate specificity and activity, determination of their expression profiles and their clustering in prognostic groups, rather than analysis of individual members, also raised a lot of interest in the last years. The existence of ABC transporters expression signatures in PDAC and their correlation with clinic-pathological characteristics of the tumours has been studied by Mohelnikova-Duchonova *et al.*^[95], and dysregulation of expression of several members of ABC family has been observed. Upregulation of ABCB4, ABCB11, ABCC1, ABCC3, ABCC5, ABCC10 and ABCG2 has been noted in PDAC, compared to non-neoplastic tissues. Surprisingly however, expression of few ABC transporters in non-neoplastic tissues also could be correlated with tumour progression and survival. Moreover, higher levels of T3 and T4 stages were associated with ABCA1 and ABCB3 upregulation and ABCG1 and ABCG2 downregulation. In contrast, smaller size tumours were connected with the cluster, in which ABCA8, ABCB5, ABCA9, ABCA10 and ABCC9 were upregulated, while downregulation of ABCA12, ABCA13, ABCC3, ABCC7 and ABCC13 has been noted. Similarly, ABCB9 and ABCC4 upregulation correlated with N1 status, while ABCA3, ABCD1 overexpression and ABCA6 and ABCC10 downregulation corresponded with increased angiogenesis.

This and previous studies demonstrated the correlation of ABC transporter expression in tumour specimens with clinic-pathological features in different cancer types^[118]. Nevertheless, the high importance of tumour microenvironment and its proposed involvement in PDAC progression, suggests that ABC transporter

Table 2 Selected ATP-binding cassette transporters responsible for the development of multi-drug resistance, their experimental inhibitors and drug specificity

ABC transporter	Inhibitor	MDR	Ref.
ABCB1	I generation: Cyclosporine A, Verapamil II generation: Valspodar, zosuquidar III generation: Tariquidar, OC144-093	Daunorubicin, epirubicin, doxorubicin, colchicines, paclitaxel, docetaxel, vincristine, vinblastine, imatinib	[46,145,146,171]
ABCC1	MK571, probenecid, ibrutinib, 3ATA	Anthracyclines, vinca alkaloids, camptothecins, daunorubicin, imatinib, etoposide, vincristine, vinblastine, methotrexate	[46,145,146,172]
ABCC2	Metothrexate, cyclosporine A, fluorescein, MK571	Doxorubicin, cisplatin, irinotecan, epirubicin, vinblastine	[46,145,146,171,173]
ABCC3	Indomethacin, sufinpyrazone, probenecid, benzmromarone	Etoposide, methotrexate, teniposide	[46,145,146,171,173]
ABCC5	Curcumin, trequensin, sildenafil	Gemcitabine, methotrexate, 6-mercaptopurine	[46,145,146,171,173]
ABCG2	Fumitremorgin C, Ko143, GF120918	Daunorubicin, doxorubicin, irinotecan, mitoxantrone, methotrexate, epirubicin, etoposide	[46,145,146,171]

MDR: Multi-drug resistance; ABC: ATP-binding cassette.

expression in non-neoplastic tissues might have important clinical implications. Following the analysis of 27 non-neoplastic pancreatic tissues and pairing them with 32 PDAC samples, 4 different clusters could be distinguished based on the gene expression profiles in cancer vs normal specimens. PN1 and PN2 clusters were characterized by upregulation of the majority of ABC transporters genes and correlated with significantly shorter patients' overall survival (OS) than patients grouped into PN3 and PN4 clusters, in which significant downregulation of genes or heterogeneous gene expression has been observed^[119]. Especially, ABCA2, ABCA4, ABCA5, ABCC2 and ABCD4 signatures showed significant difference in patients' survival when comparison between upregulated and downregulated genes was carried out. Additionally, tumour-node-metastasis, age, gender, disease stage, margin status, therapy and survival have been analysed; however, no significant correlation between those features and ABC profiles could be established. Although the study presented few limitations, such as small group size or the distance between collected tumours and control tissue, created expression clusters could be successfully implemented into clinical practice. Moreover, reduction of the analysed genes to the limited group showing most distinct expression, did not have any impact on the statistical significance of observed clinic-pathological correlations, creating more practical and convenient clinical prognostic tools.

ABC TRANSPORTERS IN CANCER THERAPY

Looking at the key role played by ABC transporters in cancer chemoresistance and the emerging knowledge on their crucial contribution to tumorigenesis, the development of targeted therapies, aiming to block or modulate their activity has become a crucial area in cancer research. Inhibition of transporter activity, arrest

of the transcription factors regulating their expression or blockade of the transporter-induced signalling pathways represent the options for impeding ABC transporters activity^[120]. So far, 3 generations of ABC transporters modulators, directed mainly against ABCB1, have been developed^[120,121] (Table 2). The first generation inhibitors, such as verapamil, quinine or cyclosporine A, compounds previously established for other conditions, in spite of promising *in vitro* activity^[122,123], showed significant toxicity, unacceptable for further usage^[123,124]. Lack of potency and specificity, combined with pharmacokinetic complications restrained their further investigation^[125]. Structural modifications of existing inhibitors, aiming to enhance their efficacy and specificity, at the same time decreasing observed adverse effects, also did not provide satisfactory results. Valspodar (cyclosporine A derivative), a second generation ABCB1 inhibitor, demonstrated enhanced efficiency accompanied by decreased toxicity^[126]. However, it showed unsatisfactory results in the majority of clinical trials, in which its co-administration with chemotherapeutics, e.g., carboplatin, paclitaxel or doxorubicin did not exhibit any benefits, and in some cases deteriorated patients' outcome^[127,128]. Likewise, application of dofequidar or biricodar citrate (VX-710)^[129] did not result to be favourable, as their use has been restricted by the potential interactions with anti-cancer therapeutics (vincristine or paclitaxel)^[130]. All these limitations led to the development of a third generation of inhibitors which potency, due to the rational QSAR design, has been described as 200-fold higher than the previously developed anti-ABCB1 molecules, greatly enhancing drug accumulation^[131]. Additionally, only minimal drug-drug interactions have been reported. Clinical trials have been commenced for zosuquidar (LY335979)^[132], elacridar (F12091)^[133], mitotane (NSC-38721)^[134], annamycin^[135] or tariquidar (XR9576)^[136]. Nevertheless disappointingly, most of the clinical trials testing their applicability have been

discontinued due to lack of significant positive response and off-site effects.

There are several reasons for the lack of success of the ABC transporters inhibition. Increased toxicity caused by off-target action in healthy tissues, as well as their high doses were the main reasons for the discontinuation of the trials for first and second generation inhibitors^[42]. Increasing evidence of substrate similarities between ABC transporters and CYP450, enzyme involved in drug metabolism, suggests interactions of tested compounds with the enzyme, which influences pharmacokinetic properties of co-administrated chemotherapeutics, changing their activity, lowering the efficacy and, as a consequence, increasing the toxicity^[137]. Therefore single-agent application of ABC transporters inhibitors should be considered in future research. Another reason for high toxicity of these modulators has been attributed to decreased clearance of anticancer agents and natural xenobiotics caused by unspecific blockade of the transporters. As an example, ABCB1 inhibition, apart from cancer cells may also result in its blockade in canalicular membrane in healthy liver or kidney, reducing the clearance of chemotherapeutics^[42,138]. The involvement of some of the ABC transporters (mostly ABCB and ABCC subfamilies) in the immune system is another obstacle, as disruption of its proper functioning may result in undesirable deterioration in anti-cancer immune responses^[139]. The ineffectiveness of targeted therapies may also lay in the functional redundancy of several ABC transporters, highly impairing full efficiency of the blockade of individual protein. Another limitation in the presented approach has been the fact that the vast majority of studies have been focused on ABCB1. Nevertheless, with increasing evidence of the role of other ABC transporters in cancer, the inhibitors of ABCC1 (e.g., probenecid, sulindac, biricodar, BAY-u9773 or MK571)^[129,140-142], ABCG2 (Ko143, fumitremorgin C, genistein, biochanin A)^[143,144] or ABCC3 (indomethacin or sulfapyrazone)^[145,146] have been considered (Table 2). However, some of them similarly to ABCB1 blockers, exhibited unfavourable toxicity levels when combined with chemotherapy. Additionally, several non-selective ABCB1 inhibitors have been tested for their activity towards other ABC transporters^[146]. Nonetheless, as the interest in ABC transporters increased only recently, the efficacy of the abovementioned therapeutic approach still needs to be evaluated. Also, the majority of the studies conducted so far have been focused on the reversal of chemoresistance rather than influencing cancer progression. However, current knowledge on the additional, or maybe principal role of ABC transporters in tumorigenesis might shed more light on the basis of current inhibitors toxicity as well as could allow for exploration of novel more specific molecules, aiming at slowing down cancer progression, rather than reversing MDR.

Considering the marginal effectiveness of ABC transporters inhibitors achieved so far, alternative concepts for ABC transporters targeting are being tested (Figure 2).

RNA interference, use of monoclonal antibodies, antisense oligonucleotides or the use of transcription regulators is currently under consideration^[147-150]. miRNA use has been also claimed as a possible way for ABC transporter regulation and reversal of chemoresistance^[151,152]. As crucial players in carcinogenesis, also confirmed in PDAC, miRNA regulation has been proposed as an interesting therapeutic tool^[153]. To date, several miRNAs have been reported to inhibit the expression of different ABC transporters, having chemotherapeutic effects^[154-156]. Moreover, it has been demonstrated that tyrosine kinase inhibitors may block ABC transporters by binding to their transmembrane domain at substrate-binding sites^[157]. Imatinib, nilotinib, sunitinib or lapatinib, drugs tested for the PDAC therapy independently of their ABC-inhibiting properties, have been demonstrated to block ABCB1, ABCC2 or ABCC10^[158-161]. However, this approach also needs further evaluation.

Currently, the use of nanoparticles for the delivery of therapeutics to the target cells has emerged as a growing area of interest^[162,163]. Their small size, together with increased surface area, enhances the stability and solubility of the administered drugs, improving their bioavailability^[164]. Additionally, controlled, prolonged release and protection from degradation present further advantages of that therapeutic approach. Co-delivery of the inhibitors of ABC transporters and chemotherapeutics with the use of nanoparticles is also applied to minimize observed side effects occurring as a result of drug-drug interactions. Nevertheless, the emerging field of the manipulation of ABC transporter activity for therapeutic purposes is still in its outset and more studies are needed to fully assess their pharmacological potential.

DISCUSSION

In the last years, ABC transporters have attracted remarkable attention of researchers from different scientific areas. The role of ABC transporters in different physiological and pathological conditions, including cancer, has been widely reported, increasing the interest in the development of their specific inhibitors. Especially, the well-known involvement of ABC transporters in the development of multi-drug resistance (MDR) led to the investigation of the potential of its reversal by blocking ABC transporter activity. Clinical relevance of several ABC transporters in multi-drug resistance reversal has been primarily attributed to P-gp, ABCG2, ABCB4 and 4 members of ABCC subfamily- ABCC1, ABCC2, ABCC3 and ABCC4^[165]. Therefore, the main focus so far has been placed on these proteins in terms of their pharmacological potential. However, in spite of the initial enthusiasm regarding ABCB1 inhibitors, their efficacy in clinical settings has failed to provide any improvements, leading to the early closure of the trials^[166,167]. Considerably high toxicity caused by lack of specificity and changes in pharmacokinetic of co-applied chemotherapeutics, decreasing their efficacy were

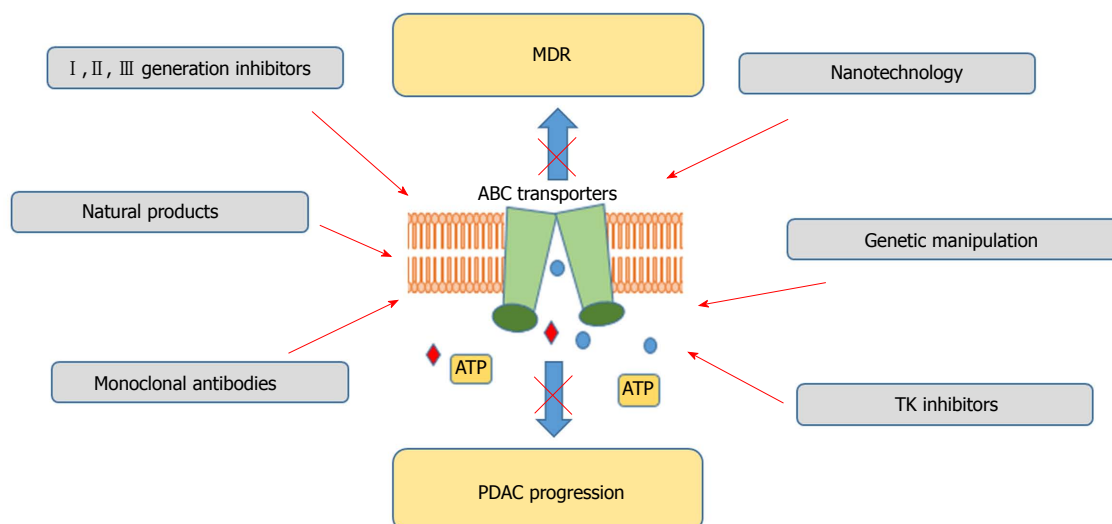


Figure 2 Pharmacological approaches towards inhibition of ATP-binding cassette transporters. MDR: Multi-drug resistance; PDAC: Pancreatic ductal adenocarcinoma; ABC: ATP-binding cassette; TK: Tyrosine kinase.

some of the reasons for the disappointing results^[168]. The successful implementation of developed inhibitors was strongly impeded by the complexity of ABC transporters functioning. The correlation between cancer chemoresistance and ABC transporters expression is two-sided and forms a specific loop, which may increase cancer resistance to applied therapies. On one hand, their expression contributes to enhanced drug efflux from the cells, diminishing their efficacy, on the other hand, many studies have reported increased expression of the transporters, induced by drugs application, complementing formed loop. Therefore, in spite of the enhancement of drug accumulation and reversal of induced chemoresistance demonstrated *in vitro*, little success has been reported during clinical trials. Also, increased toxicity and insufficient potency observed during clinical trials restrained the majority of tested compounds from the clinical use. Additionally, the majority of carried clinical trials were performed on patients previously treated with several anticancer therapeutics. Therefore, the assessment of the protein levels might have been misevaluated due to drug-induced enhancement of expression of ABC transporters. Moreover, several of the studies were designed without proper patient stratification for ABC transporters expression. As an example, little success rate in ovarian cancer patients, might be explained by low expression rate of P-gp in this tumour type^[127]. Although reversal of the drug resistance was the principal goal of ABC-targeted therapies, considering the increasing awareness of the pivotal role of ABC transporters beyond chemoresistance, their specific inhibition might not only aid to increase the activity of other therapeutics, but directly bask tumour development and progression^[56], encouraging their further exploration. Therefore, the repertoire of ABC transporters against which inhibitors are being developed should be expanded for those playing an active role, not only in MDR, but in the

expulsion of bioactive molecules. Looking at the wide variety of substrates transported by ABC transporters, together with their increased expression in cancer cells and especially cancer stem cells, the role of these proteins in the transport of signalling molecules, which activity promotes cancer progression, has become an area of interest. High impact of bioactive lipids, including phospholipids, sphingolipids or cholesterol on PDAC tumorigenesis and an emerging role of ABC transporters in their release presents a novel opportunity for targeting the disease. It has been previously demonstrated that one of the hallmarks of PDAC is lipid-dependence and that the decrease of the lipids levels may reduce cancer progression. Accordingly, aiming to block specific ABC transporters responsible for their extrusion, mainly members of ABCA and ABCC subfamilies, and depriving cancer cells of the necessary fuel may highly contribute to slowing down PDAC development. In fact, it has been demonstrated in several cancers that targeting of ABC transporters involved in lipid transport (e.g., ABCC1 in prostate or ovarian cancer or ABCC4 in neuroblastoma) showed significant improvement in *in vitro* and *in vivo* models^[75], slowing down cancer progression. Therefore, single-agent therapies based on ABC transporter inhibition should be considered to target cancer progression. Moreover, patients' treatment with ABC transporters single inhibitors would eliminate the risk of drug-drug interactions, reducing the risk of adverse events.

Importantly, the expression of ABC transporters may not only be explored in terms of their pro-tumorigenic activity but may also serve as prediction of therapy efficiency and patients' outcome. Database analysis demonstrated strong influence of the expression of the transporters e.g., ABCC3 or ABCC1 on reported 5-year survival. However, positive association of other transporters (e.g., ABCC5 or ABCA7) with the increased survival demonstrates the complexity of the role of ABC

transporters in PDAC tumorigenesis. It also shows the necessity for enhanced research in this area to fully understand and explore the therapeutic potential of these transmembrane proteins in PDAC therapy. The enhancement of chemotherapy efficacy, *e.g.*, by ABCC5 blocking, has been demonstrated for the gemcitabine-based therapies. However, considering the favourable association of this transporter with PDAC patients' survival, it is tempting to speculate that its inhibition might interfere with some of the protective functions that ABCC5 might exhibit and, as a consequence, deteriorate patients' outcome. Also, despite being overexpressed in a majority of cancer types, the role of ABC transporters is not uniform. Negative or positive correlation of the protein expression and survival observed in PDAC patients, is not invariably reflected in other cancer types. As an example ABCA7, expressed at a similar level in pancreatic and lung cancer, although positively correlated with 5-year survival in the PDAC (38% high expression vs 0% low expression), has no statistically significant effect in the latter case (48% vs 43%)^[117]. Therefore, studying the context accompanying ABC transporters expression and functioning is of high importance in order to stratify their individual members in context of their pharmacological potential in diverse cancers. Additionally, the focus of research should not be placed only on the potential of the inhibition of ABC transporters that have undermining roles in carcinogenesis. Hence, the investigation of the characteristics of the ABC transporters that favour the survival of PDAC patients should be also explored to study the mechanisms and molecules responsible for their protective function.

Finally, ABC transporters profiling in cancer has proven to provide a potent tool in estimation of patients' response to applied therapies. As an example, analysis of 21 breast cancer specimens before and after neoadjuvant treatment showed different expression of several ABC transporters^[169]. Similarly, 6 ABC transporters genes in AML samples allowed for their organization in two expression groups, correlated with resistance and patients' prognosis^[170]. Correspondingly, generation of ABC transporter expression profiles in PDAC has allowed for creation of clusters, characterized by differentiated expression of their individual members. Correlation of each cluster with a variety of disease parameters (*e.g.*, number of metastases or drug response) and more importantly, with patients' survival suggested the gene profiling for ABC transporters expression as a clinically relevant prognostic tool.

CONCLUSION

Although a lot of advancement has been achieved in the identification of new druggable targets involved in PDAC progression and chemoresistance, no significant improvement in transferring that knowledge into clinical practice has been accomplished, leaving PDAC

patients with grim prognosis. As critical players in PDAC chemoresistance and disease development, ABC transporters seem a promising target for the development of novel targeted therapies. However, despite their remarkable pharmacological potential demonstrated *in vitro*, acquired knowledge has not been successfully implemented in the clinic yet. Nevertheless, the knowledge learnt from previous mistakes and the potential reasons for the failed implementation of the inhibitors should be considered in the development of new studies and treatments. In the light of recent data, the potential of few ABC transporters beyond MDR reversal should be further explored to fully scrutinize the applicability of ABC transporter inhibition for clinical practice. More emphasis on the ABC transporters involvement in PDAC progression should be placed in prospective studies, leading to the determination of the proteins with the most pharmacological potential followed by design of single-agent treatment. The knowledge on the involvement of ABC transporters in cancer metabolic shift, their role in tumour-microenvironment cross-talk should be additionally expanded. Animal models of pancreatic cancer should be implemented in the development of new potential inhibitors to investigate their impact on abovementioned processes. In conclusion, proper study design and patients stratification regarding ABC transporters expression leading to tailored therapies should be elucidated in order to add to the efficiency of administered drugs.

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REFERENCES

- 1 ASCO.org CN. Pancreatic Cancer: Statistics. Available from: URL: <https://www.cancer.net/cancer-types/pancreatic-cancer/statistics>
- 2 Garrido-Laguna I, Hidalgo M. Pancreatic cancer: from state-of-the-art treatments to promising novel therapies. *Nat Rev Clin Oncol* 2015; **12**: 319-334 [PMID: 25824606 DOI: 10.1038/nrclinonc.2015.53]
- 3 Vaccaro V, Sperduti I, Vari S, Bria E, Melisi D, Garufi C, Nuzzo C, Scarpa A, Tortora G, Cognetti F, Reni M, Milella M. Metastatic pancreatic cancer: Is there a light at the end of the tunnel? *World J Gastroenterol* 2015; **21**: 4788-4801 [PMID: 25944992 DOI: 10.3748/wjg.v21.i16.4788]
- 4 Maitra A, Hruban RH. Pancreatic cancer. *Annu Rev Pathol* 2008; **3**: 157-188 [PMID: 18039136 DOI: 10.1146/annurev.pathmechdis.3.121806.154305]
- 5 Yachida S, Iacobuzio-Donahue CA. Evolution and dynamics of pancreatic cancer progression. *Oncogene* 2013; **32**: 5253-5260

- [PMID: 23416985 DOI: 10.1038/onc.2013.29]
- 6 **Abel EV**, Simeone DM. Biology and clinical applications of pancreatic cancer stem cells. *Gastroenterology* 2013; **144**: 1241-1248 [PMID: 23622133 DOI: 10.1053/j.gastro.2013.01.072]
- 7 **Wang S**, Huang S, Sun YL. Epithelial-Mesenchymal Transition in Pancreatic Cancer: A Review. *Biomed Res Int* 2017; **2017**: 2646148 [PMID: 29379795 DOI: 10.1155/2017/2646148]
- 8 **Feig C**, Gopinathan A, Neesse A, Chan DS, Cook N, Tuveson DA. The pancreas cancer microenvironment. *Clin Cancer Res* 2012; **18**: 4266-4276 [PMID: 22896693 DOI: 10.1158/1078-0432.ccr-11-3114]
- 9 **Charles N**, Ozawa T, Squatrito M, Bleau AM, Brennan CW, Hambarzumyan D, Holland EC. Perivascular nitric oxide activates notch signaling and promotes stem-like character in PDGF-induced glioma cells. *Cell Stem Cell* 2010; **6**: 141-152 [PMID: 20144787 DOI: 10.1016/j.stem.2010.01.001]
- 10 **Adamska A**, Domenichini A, Falasca M. Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies. *Int J Mol Sci* 2017; **18**: pii: E1338 [PMID: 28640192 DOI: 10.3390/ijms18071338]
- 11 **Burris HA 3rd**, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; **15**: 2403-2413 [PMID: 9196156 DOI: 10.1200/JCO.1997.15.6.2403]
- 12 **Conroy T**, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardière C, Bannoun J, Bachet JB, Khemissa-Akouz F, Péré-Vergé D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M; Groupe Tumeurs Digestives of Unicancer; PRODIGE Intergrup. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med* 2011; **364**: 1817-1825 [PMID: 21561347 DOI: 10.1056/NEJMoa1011923]
- 13 **Von Hoff DD**, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulander SA, Ma WW, Saleh MN, Harris M, Reni M, Dowden S, Laheru D, Bahary N, Ramanathan RK, Tabernero J, Hidalgo M, Goldstein D, Van Cutsem E, Wei X, Iglesias J, Renschler MF. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med* 2013; **369**: 1691-1703 [PMID: 24131140 DOI: 10.1056/NEJMoa1304369]
- 14 **Moore MJ**, Goldstein D, Hamm J, Figier A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W; National Cancer Institute of Canada Clinical Trials Group. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2007; **25**: 1960-1966 [PMID: 17452677 DOI: 10.1200/jco.2006.07.9525]
- 15 **Li Y**, Sun J, Jiang Z, Zhang L, Liu G. Gemcitabine and S-1 combination chemotherapy versus gemcitabine alone for locally advanced and metastatic pancreatic cancer: a meta-analysis of randomized controlled trials in Asia. *J Chemother* 2015; **27**: 227-234 [PMID: 25790948 DOI: 10.1179/1973947815y.0000000013]
- 16 **Kindler HL**, Ioka T, Richel DJ, Bannoun J, Létoirneau R, Okusaka T, Funakoshi A, Furuse J, Park YS, Ohkawa S, Springett GM, Wasan HS, Trask PC, Bycott P, Ricart AD, Kim S, Van Cutsem E. Axitinib plus gemcitabine versus placebo plus gemcitabine in patients with advanced pancreatic adenocarcinoma: a double-blind randomised phase 3 study. *Lancet Oncol* 2011; **12**: 256-262 [PMID: 21306953 DOI: 10.1016/s1470-2045(11)70004-3]
- 17 **Herrmann R**, Bodoky G, Ruhstaller T, Glimelius B, Bajetta E, Schüller J, Saletti P, Bauer J, Figier A, Pestalozzi B, Köhne CH, Mingrone W, Stemmer SM, Tamas K, Kornek GV, Koeberle D, Cina S, Bernhard J, Dietrich D, Scheithauer W; Swiss Group for Clinical Cancer Research; Central European Cooperative Oncology Group. Gemcitabine plus capecitabine compared with gemcitabine alone in advanced pancreatic cancer: a randomized, multicenter, phase III trial of the Swiss Group for Clinical Cancer Research and the Central European Cooperative Oncology Group. *J Clin Oncol* 2007; **25**: 2212-2217 [PMID: 17538165 DOI: 10.1200/jco.2006.09.0886]
- 18 Momenta Pharmaceuticals Inc. M402 in combination with nab-Paclitaxel and gemcitabine in pancreatic cancer. 2017 In: ClinicalTrials.gov [Internet]. Available from: <https://clinicaltrials.gov/ct2/show/NCT01621243>. Accessed on: 15.05.2017
- 19 **Trédan O**, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. *J Natl Cancer Inst* 2007; **99**: 1441-1454 [PMID: 17895480 DOI: 10.1093/jnci/djm135]
- 20 **DI C**, Zhao Y. Multiple drug resistance due to resistance to stem cells and stem cell treatment progress in cancer (Review). *Exp Ther Med* 2015; **9**: 289-293 [PMID: 25574188 DOI: 10.3892/etm.2014.2141]
- 21 **Durand RE**, Olive PL. Resistance of tumor cells to chemo- and radiotherapy modulated by the three-dimensional architecture of solid tumors and spheroids. *Methods Cell Biol* 2001; **64**: 211-233 [PMID: 11070841 DOI: 10.1016/S0091-679X(01)64015-9]
- 22 **Liu YY**, Han TY, Giuliano AE, Cabot MC. Ceramide glycosylation potentiates cellular multidrug resistance. *FASEB J* 2001; **15**: 719-730 [PMID: 11259390 DOI: 10.1096/fj.00-0223com]
- 23 **Jain RK**. Delivery of molecular and cellular medicine to solid tumors. *Adv Drug Deliv Rev* 2012; **64**: 353-365 [PMID: 24511174 DOI: 10.1016/j.addr.2012.09.011]
- 24 **Gillet JP**, Gottesman MM. Overcoming multidrug resistance in cancer: 35 years after the discovery of ABCB1. *Drug Resist Updat* 2012; **15**: 2-4 [PMID: 22465109 DOI: 10.1016/j.drug.2012.03.001]
- 25 **Gottesman MM**, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002; **2**: 48-58 [PMID: 11902585 DOI: 10.1038/nrc706]
- 26 **Higgins CF**. ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* 1992; **8**: 67-113 [PMID: 1282354 DOI: 10.1146/annurev.cb.08.110192.000435]
- 27 **Lage H**. An overview of cancer multidrug resistance: a still unsolved problem. *Cell Mol Life Sci* 2008; **65**: 3145-3167 [PMID: 18581055 DOI: 10.1007/s00018-008-8111-5]
- 28 **Shukla S**, Ohnuma S, Ambudkar SV. Improving cancer chemotherapy with modulators of ABC drug transporters. *Curr Drug Targets* 2011; **12**: 621-630 [PMID: 21039338 DOI: 10.2174/138945011795378540]
- 29 **Dean M**. The genetics of ATP-binding cassette transporters. *Methods Enzymol* 2005; **400**: 409-429 [PMID: 16399363 DOI: 10.1016/s0076-6879(05)00024-8]
- 30 **Vasilou V**, Vasilou K, Nebert DW. Human ATP-binding cassette (ABC) transporter family. *Hum Genomics* 2009; **3**: 281-290 [PMID: 19403462 DOI: 10.1186/1479-7364-3-3-281]
- 31 **Locher KP**. Structure and mechanism of ABC transporters. *Curr Opin Struct Biol* 2004; **14**: 426-431 [PMID: 15313236 DOI: 10.1016/j.sbi.2004.06.005]
- 32 **Linton KJ**. Structure and function of ABC transporters. *Physiology* (Bethesda) 2007; **22**: 122-130 [PMID: 17420303 DOI: 10.1152/physiol.00046.2006]
- 33 **Hyde SC**, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, Gallagher MP, Gill DR, Hubbard RE, Higgins CF. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* 1990; **346**: 362-365 [PMID: 1973824 DOI: 10.1038/346362a0]
- 34 **Ambudkar SV**, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu Rev Pharmacol Toxicol* 1999; **39**: 361-398 [PMID: 10331089 DOI: 10.1146/annurev.pharmtox.39.1.361]
- 35 **Albrecht C**, Vitorro E. The ABCA subfamily--gene and protein structures, functions and associated hereditary diseases. *Pflugers Arch* 2007; **453**: 581-589 [PMID: 16586097 DOI: 10.1007/s00424-006-0047-8]
- 36 **Takahashi K**, Kimura Y, Nagata K, Yamamoto A, Matsuo M, Ueda K. ABC proteins: key molecules for lipid homeostasis. *Med Mol Morphol* 2005; **38**: 2-12 [PMID: 16158173 DOI: 10.1007/s00795-004-0278-8]

- 37 **Dean M**, Annilo T. Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates. *Annu Rev Genomics Hum Genet* 2005; **6**: 123-142 [PMID: 16124856 DOI: 10.1146/annurev.genom.6.080604.162122]
- 38 **Esser L**, Zhou F, Pluchino KM, Shiloach J, Ma J, Tang WK, Gutierrez C, Zhang A, Shukla S, Madigan JP, Zhou T, Kwong PD, Ambudkar SV, Gottesman MM, Xia D. Structures of the Multidrug Transporter P-glycoprotein Reveal Asymmetric ATP Binding and the Mechanism of Polyspecificity. *J Biol Chem* 2017; **292**: 446-461 [PMID: 27864369 DOI: 10.1074/jbc.M116.755884]
- 39 **Callaghan R**. Providing a molecular mechanism for P-glycoprotein; why would I bother? *Biochem Soc Trans* 2015; **43**: 995-1002 [PMID: 26517914 DOI: 10.1042/BST20150131]
- 40 **Sharom FJ**. The P-glycoprotein multidrug transporter. *Essays Biochem* 2011; **50**: 161-178 [PMID: 21967057 DOI: 10.1042/bse0500161]
- 41 **Didziapetris R**, Japertas P, Avdeef A, Petrauskas A. Classification analysis of P-glycoprotein substrate specificity. *J Drug Target* 2003; **11**: 391-406 [PMID: 15203928 DOI: 10.1080/1061186031001648248]
- 42 **Ueda K**. ABC proteins protect the human body and maintain optimal health. *Biosci Biotechnol Biochem* 2011; **75**: 401-409 [PMID: 21389634 DOI: 10.1271/bbb.100816]
- 43 **Soucek P**, Hlavac V, Elsnerova K, Vavlikova R, Kozevnikova R, Raus K. Whole exome sequencing analysis of ABCB8 and ABCD2 genes associating with clinical course of breast carcinoma. *Physiol Res* 2015; **64** Suppl 4: S549-S557 [PMID: 26681085]
- 44 **Cordon-Cardo C**, O'Brien JP, Boccia J, Casals D, Bertino JR, Melamed MR. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *J Histochem Cytochem* 1990; **38**: 1277-1287 [PMID: 1974900 DOI: 10.1177/38.9.1974900]
- 45 **Sarkadi B**, Homolya L, Szakacs G, Váradi A. Human multidrug resistance ABCB and ABCG transporters: participation in a chemoinnate defense system. *Physiol Rev* 2006; **86**: 1179-1236 [PMID: 17015488 DOI: 10.1152/physrev.00037.2005]
- 46 **Schinkel AH**, Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* 2003; **55**: 3-29 [PMID: 12535572 DOI: 10.1016/S0169-409X(02)00169-2]
- 47 **Kruh GD**, Belinsky MG. The MRP family of drug efflux pumps. *Oncogene* 2003; **22**: 7537-7552 [PMID: 14576857 DOI: 10.1038/sj.onc.1206953]
- 48 **Juliano RL**, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* 1976; **455**: 152-162 [PMID: 990323 DOI: 10.1016/0005-2736(76)90160-7]
- 49 **Riordan JR**, Ling V. Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability. *J Biol Chem* 1979; **254**: 12701-12705 [PMID: 500733]
- 50 **Sharom FJ**, Liu R, Qu Q, Romsicki Y. Exploring the structure and function of the P-glycoprotein multidrug transporter using fluorescence spectroscopic tools. *Semin Cell Dev Biol* 2001; **12**: 257-265 [PMID: 11428918 DOI: 10.1006/scdb.2000.0251]
- 51 **Borgnia MJ**, Eytan GD, Assaraf YG. Competition of hydrophobic peptides, cytotoxic drugs, and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its ATPase activity. *J Biol Chem* 1996; **271**: 3163-3171 [PMID: 8621716 DOI: 10.1074/jbc.271.6.3163]
- 52 **Callaghan R**, Crowley E, Potter S, Kerr ID. P-glycoprotein: so many ways to turn it on. *J Clin Pharmacol* 2008; **48**: 365-378 [PMID: 18156365 DOI: 10.1177/0091270007311568]
- 53 **Penson RT**, Oliva E, Skates SJ, Glyptis T, Fuller AF Jr, Goodman A, Seiden MV. Expression of multidrug resistance-1 protein inversely correlates with paclitaxel response and survival in ovarian cancer patients: a study in serial samples. *Gynecol Oncol* 2004; **93**: 98-106 [PMID: 15047220 DOI: 10.1016/j.ygyno.2003.11.053]
- 54 **Han K**, Kahng J, Kim M, Lim J, Kim Y, Cho B, Kim HK, Min WS, Kim CC, Lee KY, Kim BK, Kang CS. Expression of functional markers in acute nonlymphoblastic leukemia. *Acta Haematol* 2000; **104**: 174-180 [PMID: 11279307 DOI: 10.1159/000046511]
- 55 **Chung HC**, Rha SY, Kim JH, Roh JK, Min JS, Lee KS, Kim BS, Lee KB. P-glycoprotein: the intermediate end point of drug response to induction chemotherapy in locally advanced breast cancer. *Breast Cancer Res Treat* 1997; **42**: 65-72 [PMID: 9116319 DOI: 10.1023/a:1005739525196]
- 56 **Teodori E**, Dei S, Martelli C, Scapecchi S, Gualtieri F. The functions and structure of ABC transporters: implications for the design of new inhibitors of Pgp and MRP1 to control multidrug resistance (MDR). *Curr Drug Targets* 2006; **7**: 893-909 [PMID: 16842220 DOI: 10.2174/138945006777709520]
- 57 **Avendaño C**, Menéndez JC. Inhibitors of multidrug resistance to antitumor agents (MDR). *Curr Med Chem* 2002; **9**: 159-193 [PMID: 11860354 DOI: 10.2174/0929867023371175]
- 58 **Ueda K**, Cardarelli C, Gottesman MM, Pastan I. Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc Natl Acad Sci USA* 1987; **84**: 3004-3008 [PMID: 3472246 DOI: 10.1073/pnas.84.9.3004]
- 59 **Borst P**, Evers R, Koel M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst* 2000; **92**: 1295-1302 [PMID: 10944550 DOI: 10.1093/jnci/92.16.1295]
- 60 **Berger W**, Setinek U, Hollaus P, Zidek T, Steiner E, Elbling L, Cantonati H, Attems J, Gsur A, Micksche M. Multidrug resistance markers P-glycoprotein, multidrug resistance protein 1, and lung resistance protein in non-small cell lung cancer: prognostic implications. *J Cancer Res Clin Oncol* 2005; **131**: 355-363 [PMID: 15856298 DOI: 10.1007/s00432-004-0653-9]
- 61 **Zurita AJ**, Diestra JE, Condom E, Garcia Del Muro X, Scheffer GL, Scheper RJ, Pérez J, Germá-Lluch JR, Izquierdo MA. Lung resistance-related protein as a predictor of clinical outcome in advanced testicular germ-cell tumours. *Br J Cancer* 2003; **88**: 879-886 [PMID: 12644825 DOI: 10.1038/sj.bjc.6600803]
- 62 **Kruh GD**, Zeng H, Rea PA, Liu G, Chen ZS, Lee K, Belinsky MG. MRP subfamily transporters and resistance to anticancer agents. *J Bioenerg Biomembr* 2001; **33**: 493-501 [PMID: 11804191 DOI: 10.1023/a:1012827221844]
- 63 **Schuetz JD**, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, Kumar A, Fridland A. MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nat Med* 1999; **5**: 1048-1051 [PMID: 10470083 DOI: 10.1038/12487]
- 64 **Reid G**, Wielinga P, Zelcer N, De Haas M, Van Deemter L, Wijnholds J, Balzarini J, Borst P. Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. *Mol Pharmacol* 2003; **63**: 1094-1103 [PMID: 12695538 DOI: 10.1124/mol.63.5.1094]
- 65 **Noguchi K**, Katayama K, Mitsuhashi J, Sugimoto Y. Functions of the breast cancer resistance protein (BCRP/ABCG2) in chemotherapy. *Adv Drug Deliv Rev* 2009; **61**: 26-33 [PMID: 19111841 DOI: 10.1016/j.addr.2008.07.003]
- 66 **Mao Q**, Unadkat JD. Role of the breast cancer resistance protein (ABCG2) in drug transport. *AAPS J* 2005; **7**: E118-E133 [PMID: 16146333 DOI: 10.1208/aapsj070112]
- 67 **Doyle LA**, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, Ross DD. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci USA* 1998; **95**: 15665-15670 [PMID: 9861027]
- 68 **Allikmets R**, Schriml LM, Hutchinson A, Romano-Spica V, Dean M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res* 1998; **58**: 5337-5339 [PMID: 9850061]
- 69 **Scharenberg CW**, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 2002; **99**: 507-512 [PMID: 11781231 DOI: 10.1182/blood.V99.2.507]
- 70 **Santamarina-Fojo S**, Remaley AT, Neufeld EB, Brewer HB Jr. Regulation and intracellular trafficking of the ABCA1 transporter.

- J Lipid Res* 2001; **42**: 1339-1345 [PMID: 11518753]
- 71 **Oram JF**, Vaughan AM. ABCA1-mediated transport of cellular cholesterol and phospholipids to HDL apolipoproteins. *Curr Opin Lipidol* 2000; **11**: 253-260 [PMID: 10882340]
- 72 **Ruban EL**, Ferro R, Arifin SA, Falasca M. Lysophosphatidylinositol: a novel link between ABC transporters and G-protein-coupled receptors. *Biochem Soc Trans* 2014; **42**: 1372-1377 [PMID: 25233417 DOI: 10.1042/bst20140151]
- 73 **Piñeiro R**, Maffucci T, Falasca M. The putative cannabinoid receptor GPR55 defines a novel autocrine loop in cancer cell proliferation. *Oncogene* 2011; **30**: 142-152 [PMID: 20838378 DOI: 10.1038/onc.2010.417]
- 74 **Mitra P**, Oskeritzian CA, Payne SG, Beaven MA, Milstien S, Spiegel S. Role of ABC1 in export of sphingosine-1-phosphate from mast cells. *Proc Natl Acad Sci U S A* 2006; **103**: 16394-16399 [PMID: 17050692 DOI: 10.1073/pnas.0603734103]
- 75 **Fletcher JI**, Haber M, Henderson MJ, Norris MD. ABC transporters in cancer: more than just drug efflux pumps. *Nat Rev Cancer* 2010; **10**: 147-156 [PMID: 20075923 DOI: 10.1038/nrc2789]
- 76 **König J**, Hartel M, Nies AT, Martignoni ME, Guo J, Büchler MW, Friess H, Keppler D. Expression and localization of human multidrug resistance protein (ABCC) family members in pancreatic carcinoma. *Int J Cancer* 2005; **115**: 359-367 [PMID: 15688370 DOI: 10.1002/ijc.20831]
- 77 **Scheffer GL**, Kool M, de Haas M, de Vree JM, Pijnenborg AC, Bosman DK, Elferink RP, van der Valk P, Borst P, Scheper RJ. Tissue distribution and induction of human multidrug resistant protein 3. *Lab Invest* 2002; **82**: 193-201 [PMID: 11850532]
- 78 **Hagmann W**, Jesnowski R, Faissner R, Guo C, Löhr JM. ATP-binding cassette C transporters in human pancreatic carcinoma cell lines. Upregulation in 5-fluorouracil-resistant cells. *Pancreatology* 2009; **9**: 136-144 [PMID: 19077464 DOI: 10.1159/000178884]
- 79 **Hagmann W**, Faissner R, Schnölzer M, Löhr M, Jesnowski R. Membrane drug transporters and chemoresistance in human pancreatic carcinoma. *Cancers (Basel)* 2010; **3**: 106-125 [PMID: 24212609 DOI: 10.3390/cancers3010106]
- 80 **Hirohashi T**, Suzuki H, Sugiyama Y. Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). *J Biol Chem* 1999; **274**: 15181-15185 [PMID: 10329726 DOI: 10.1074/jbc.274.21.15181]
- 81 **Zeng H**, Liu G, Rea PA, Kruh GD. Transport of amphipathic anions by human multidrug resistance protein 3. *Cancer Res* 2000; **60**: 4779-4784 [PMID: 10987286]
- 82 **Kool M**, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Elferink RP, Baas F, Borst P. MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci USA* 1999; **96**: 6914-6919 [PMID: 10359813 DOI: 10.1073/pnas.96.12.6914]
- 83 **Hagmann W**, Jesnowski R, Löhr JM. Interdependence of gemcitabine treatment, transporter expression, and resistance in human pancreatic carcinoma cells. *Neoplasia* 2010; **12**: 740-747 [PMID: 20824050 DOI: 10.1593/neo.10576]
- 84 **Nambaru PK**, Hübner T, Köck K, Mews S, Grube M, Payen L, Guillon J, Sendler M, Jedlitschky G, Rimmbach C, Rosskopf D, Kowalczyk DW, Kroemer HK, Weiss FU, Mayerle J, Lerch MM, Ritter CA. Drug efflux transporter multidrug resistance-associated protein 5 affects sensitivity of pancreatic cancer cell lines to the nucleoside anticancer drug 5-fluorouracil. *Drug Metab Dispos* 2011; **39**: 132-139 [PMID: 20930123 DOI: 10.1124/dmd.110.033613]
- 85 **Falasca M**, Kim M, Casari I. Pancreatic cancer: Current research and future directions. *Biochim Biophys Acta* 2016; **1865**: 123-132 [PMID: 26794394 DOI: 10.1016/j.bbcan.2016.01.001]
- 86 **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]
- 87 **Sousa CM**, Kimmelman AC. The complex landscape of pancreatic cancer metabolism. *Carcinogenesis* 2014; **35**: 1441-1450 [PMID: 24743516 DOI: 10.1093/carcin/bgu097]
- 88 **Ying H**, Kimmelman AC, Lyssiotis CA, Hua S, Chu GC, Fletcher-Sananikone E, Locasale JW, Son J, Zhang H, Colloff JL, Yan H, Wang W, Chen S, Viale A, Zheng H, Paik JH, Lim C, Guimaraes AR, Martin ES, Chang J, Hezel AF, Perry SR, Hu J, Gan B, Xiao Y, Asara JM, Weissleder R, Wang YA, Chin L, Cantley LC, DePinho RA. Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell* 2012; **149**: 656-670 [PMID: 22541435 DOI: 10.1016/j.cell.2012.01.058]
- 89 **Son J**, Lyssiotis CA, Ying H, Wang X, Hua S, Ligorio M, Perera RM, Ferrone CR, Mullarky E, Shyh-Chang N, Kang Y, Fleming JB, Bardeesy N, Asara JM, Haigis MC, DePinho RA, Cantley LC, Kimmelman AC. Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature* 2013; **496**: 101-105 [PMID: 23535601 DOI: 10.1038/nature12040]
- 90 **Swierczynski J**, Hebanowska A, Sledzinski T. Role of abnormal lipid metabolism in development, progression, diagnosis and therapy of pancreatic cancer. *World J Gastroenterol* 2014; **20**: 2279-2303 [PMID: 24605027 DOI: 10.3748/wjg.v20.i9.2279]
- 91 **Kamphorst JJ**, Cross JR, Fan J, de Stanchina E, Mathew R, White EP, Thompson CB, Rabinowitz JD. Hypoxic and Ras-transformed cells support growth by scavenging unsaturated fatty acids from lysophospholipids. *Proc Natl Acad Sci USA* 2013; **110**: 8882-8887 [PMID: 23671091 DOI: 10.1073/pnas.1307237110]
- 92 **Rysman E**, Brusselmans K, Scheys K, Timmermans L, Derua R, Munck S, Van Veldhoven PP, Waltregny D, Daniëls VW, Machiels J, Vanderhoydonc F, Smans K, Waelkens E, Verhoeven G, Swinnen JV. De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation. *Cancer Res* 2010; **70**: 8117-8126 [PMID: 20876798 DOI: 10.1158/0008-5472.can-09-3871]
- 93 **Baenke F**, Peck B, Miess H, Schulze A. Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development. *Dis Model Mech* 2013; **6**: 1353-1363 [PMID: 24203995 DOI: 10.1242/dmm.011338]
- 94 **Guillaumond F**, Bidaut G, Ouaisi M, Servais S, Gouirand V, Olivares O, Lac S, Borge L, Roques J, Gayet O, Pinault M, Guimaraes C, Nigri J, Loncle C, Lavaut MN, Garcia S, Tailleux A, Staels B, Calvo E, Tomasini R, Iovanna JL, Vasseur S. Cholesterol uptake disruption, in association with chemotherapy, is a promising combined metabolic therapy for pancreatic adenocarcinoma. *Proc Natl Acad Sci USA* 2015; **112**: 2473-2478 [PMID: 25675507 DOI: 10.1073/pnas.1421601112]
- 95 **Mohelnikova-Duchonova B**, Brynychova V, Oliverius M, Honsova E, Kala Z, Muckova K, Soucek P. Differences in transcript levels of ABC transporters between pancreatic adenocarcinoma and nonneoplastic tissues. *Pancreas* 2013; **42**: 707-716 [PMID: 23462326 DOI: 10.1097/MPA.0b013e318279b861]
- 96 **Mantovani A**, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008; **454**: 436-444 [PMID: 18650914 DOI: 10.1038/nature07205]
- 97 **Tessner TG**, Muhale F, Riehl TE, Anant S, Stenson WF. Prostaglandin E2 reduces radiation-induced epithelial apoptosis through a mechanism involving AKT activation and bax translocation. *J Clin Invest* 2004; **114**: 1676-1685 [PMID: 15578100 DOI: 10.1172/jci22218]
- 98 **Reid G**, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J, Borst P. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc Natl Acad Sci USA* 2003; **100**: 9244-9249 [PMID: 12835412 DOI: 10.1073/pnas.1033060100]
- 99 **Knab LM**, Grippo PJ, Bentrem DJ. Involvement of eicosanoids in the pathogenesis of pancreatic cancer: the roles of cyclooxygenase-2 and 5-lipoxygenase. *World J Gastroenterol* 2014; **20**: 10729-10739 [PMID: 25152576 DOI: 10.3748/wjg.v20.i31.10729]
- 100 **Hussain SP**, Hofseth LJ, Harris CC. Radical causes of cancer. *Nat Rev Cancer* 2003; **3**: 276-285 [PMID: 12671666 DOI: 10.1038/nrc1046]
- 101 **Meister A**. Selective modification of glutathione metabolism. *Science* 1983; **220**: 472-477 [PMID: 6836290 DOI: 10.1126/

- science.6836290]
- 102 **Liesa M**, Qiu W, Shirihai OS. Mitochondrial ABC transporters function: the role of ABCB10 (ABC-me) as a novel player in cellular handling of reactive oxygen species. *Biochim Biophys Acta* 2012; **1823**: 1945-1957 [PMID: 22884976 DOI: 10.1016/j.bbamer.2012.07.013]
- 103 **Demico EG**, Kavanagh KT, Romieu-Mourez R, Wang X, Shin SR, Landesman-Bollag E, Seldin DC, Sonenshein GE. RelB/p52 NF-kappaB complexes rescue an early delay in mammary gland development in transgenic mice with targeted superrepressor IkappaB-alpha expression and promote carcinogenesis of the mammary gland. *Mol Cell Biol* 2005; **25**: 10136-10147 [PMID: 16260626 DOI: 10.1128/mcb.25.22.10136-10147.2005]
- 104 **Wang X**, Campos CR, Peart JC, Smith LK, Boni JL, Cannon RE, Miller DS. Nrf2 upregulates ATP binding cassette transporter expression and activity at the blood-brain and blood-spinal cord barriers. *J Neurosci* 2014; **34**: 8585-8593 [PMID: 24948812 DOI: 10.1523/jneurosci.2935-13.2014]
- 105 **Cabello CM**, Bair WB 3rd, Wondrak GT. Experimental therapeutics: targeting the redox Achilles heel of cancer. *Curr Opin Investig Drugs* 2007; **8**: 1022-1037 [PMID: 18058573]
- 106 **Olive KP**, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, Madhu B, Goldgraben MA, Caldwell ME, Allard D, Frese KK, Denicola G, Feig C, Combs C, Winter SP, Ireland-Zecchini H, Reichelt S, Howat WJ, Chang A, Dhara M, Wang L, Rückert F, Grützmann R, Pilarsky C, Izeradjene K, Hingorani SR, Huang P, Davies SE, Plunkett W, Egorin M, Hruban RH, Whitebread N, McGovern K, Adams J, Iacobuzio-Donahue C, Griffiths J, Tuveson DA. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* 2009; **324**: 1457-1461 [PMID: 19460966 DOI: 10.1126/science.1171362]
- 107 Olive K. Clinical Trial: IPI-926-03 for metastatic pancreatic ductal adenocarcinoma patients who have not been treated with other chemotherapy. Available from: <https://www.olivelab.org/ipi-926-03.html>. Accessed on: 17.04.2018
- 108 **Hingorani SR**, Harris WP, Beck JT, Berdov BA, Wagner SA, Pshevolotsky EM, Tjulandini SA, Gladkov OA, Holcombe RF, Korn R, Raghunand N, Dychter S, Jiang P, Shepard HM, Devoe CE. Phase Ib Study of PEGylated Recombinant Human Hyaluronidase and Gemcitabine in Patients with Advanced Pancreatic Cancer. *Clin Cancer Res* 2016; **22**: 2848-2854 [PMID: 26813359 DOI: 10.1158/1078-0432.ccr-15-2010]
- 109 **Moreau A**, Le Vee M, Jouan E, Parmentier Y, Fardel O. Drug transporter expression in human macrophages. *Fundam Clin Pharmacol* 2011; **25**: 743-752 [PMID: 21210849 DOI: 10.1111/j.1472-8206.2010.00913.x]
- 110 **Vander Borcht S**, Komuta M, Libbrecht L, Katoonizadeh A, Aerts R, Dymarkowski S, Verslype C, Nevens F, Roskams T. Expression of multidrug resistance-associated protein 1 in hepatocellular carcinoma is associated with a more aggressive tumour phenotype and may reflect a progenitor cell origin. *Liver Int* 2008; **28**: 1370-1380 [PMID: 19055643 DOI: 10.1111/j.1478-3231.2008.01889.x]
- 111 **Weinstein RS**, Jakate SM, Dominguez JM, Lebovitz MD, Koukoulis GK, Kuzak JR, Klusens LF, Grogan TM, Saclarides TJ, Roninson IB. Relationship of the expression of the multidrug resistance gene product (P-glycoprotein) in human colon carcinoma to local tumor aggressiveness and lymph node metastasis. *Cancer Res* 1991; **51**: 2720-2726 [PMID: 1673639]
- 112 **Filipits M**, Suchomel RW, Dekan G, Haider K, Valdimarsson G, Depisch D, Pirker R. MRP and MDR1 gene expression in primary breast carcinomas. *Clin Cancer Res* 1996; **2**: 1231-1237 [PMID: 9816292]
- 113 **Lee CJ**, Li C, Simeone DM. Human pancreatic cancer stem cells: implications for how we treat pancreatic cancer. *Transl Oncol* 2008; **1**: 14-18 [PMID: 18607507 DOI: 10.1593/tlo.08013]
- 114 **Li C**, Lee CJ, Simeone DM. Identification of human pancreatic cancer stem cells. *Methods Mol Biol* 2009; **568**: 161-173 [PMID: 19582426 DOI: 10.1007/978-1-59745-280-9_10]
- 115 **Dean M**, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer* 2005; **5**: 275-284 [PMID: 15803154 DOI: 10.1038/nrc1590]
- 116 **Barbet R**, Peiffer I, Hutchins JR, Hatzfeld A, Garrido E, Hatzfeld JA. Expression of the 49 human ATP binding cassette (ABC) genes in pluripotent embryonic stem cells and in early- and late-stage multipotent mesenchymal stem cells: possible role of ABC plasma membrane transporters in maintaining human stem cell pluripotency. *Cell Cycle* 2012; **11**: 1611-1620 [PMID: 22456339 DOI: 10.4161/cc.20023]
- 117 The Human Protein Atlas. Available from: <https://www.proteinatlas.org/>. Accessed on 18.04.2018
- 118 **Dvorak P**, Pesta M, Soucek P. ABC gene expression profiles have clinical importance and possibly form a new hallmark of cancer. *Tumour Biol* 2017; **39**: 1010428317699800 [PMID: 28468577 DOI: 10.1177/1010428317699800]
- 119 **Dvorak P**, Hlavac V, Mohelnikova-Duchonova B, Liska V, Pesta M, Soucek P. Downregulation of ABC Transporters in Non-neoplastic Tissues Confers Better Prognosis for Pancreatic and Colorectal Cancer Patients. *J Cancer* 2017; **8**: 1959-1971 [PMID: 28819395 DOI: 10.7150/jca.19364]
- 120 **Kathawala RJ**, Gupta P, Ashby CR Jr, Chen ZS. The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. *Drug Resist Updat* 2015; **18**: 1-17 [PMID: 25554624 DOI: 10.1016/j.drup.2014.11.002]
- 121 **Falasca M**, Linton KJ. Investigational ABC transporter inhibitors. *Expert Opin Investig Drugs* 2012; **21**: 657-666 [PMID: 22493979 DOI: 10.1517/13543784.2012.679339]
- 122 **Tsuruo T**, Iida H, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res* 1981; **41**: 1967-1972 [PMID: 7214365]
- 123 **Kelly RJ**, Draper D, Chen CC, Robey RW, Figg WD, Piekarz RL, Chen X, Gardner ER, Balis FM, Venkatesan AM, Steinberg SM, Fojo T, Bates SE. A pharmacodynamic study of docetaxel in combination with the P-glycoprotein antagonist tariquidar (XR9576) in patients with lung, ovarian, and cervical cancer. *Clin Cancer Res* 2011; **17**: 569-580 [PMID: 21081657 DOI: 10.1158/1078-0432.ccr-10-1725]
- 124 **Pennock GD**, Dalton WS, Roeske WR, Appleton CP, Mosley K, Plezia P, Miller TP, Salmon SE. Systemic toxic effects associated with high-dose verapamil infusion and chemotherapy administration. *J Natl Cancer Inst* 1991; **83**: 105-110 [PMID: 1988684 DOI: 10.1093/jnci/83.2.105]
- 125 **Yu M**, Ocana A, Tannock IF. Reversal of ATP-binding cassette drug transporter activity to modulate chemoresistance: why has it failed to provide clinical benefit? *Cancer Metastasis Rev* 2013; **32**: 211-227 [PMID: 23093326 DOI: 10.1007/s10555-012-9402-8]
- 126 **Tidefelt U**, Liliemark J, Gruber A, Liliemark E, Sundman-Engberg B, Juliusson G, Stenke L, Elmhorn-Rosenborg A, Möllgård L, Lehman S, Xu D, Covelli A, Gustavsson B, Paul C. P-Glycoprotein inhibitor valspodar (PSC 833) increases the intracellular concentrations of daunorubicin in vivo in patients with P-glycoprotein-positive acute myeloid leukemia. *J Clin Oncol* 2000; **18**: 1837-1844 [PMID: 10784624 DOI: 10.1200/jco.2000.18.9.1837]
- 127 **Lhommé C**, Joly F, Walker JL, Lissoni AA, Nicoletto MO, Manikhas GM, Baekelandt MM, Gordon AN, Fracasso PM, Mielowski WL, Jones GJ, Dugan MH. Phase III study of valspodar (PSC 833) combined with paclitaxel and carboplatin compared with paclitaxel and carboplatin alone in patients with stage IV or suboptimally debulked stage III epithelial ovarian cancer or primary peritoneal cancer. *J Clin Oncol* 2008; **26**: 2674-2682 [PMID: 18509179 DOI: 10.1200/jco.2007.14.9807]
- 128 **Greenberg PL**, Lee SJ, Advani R, Tallman MS, Sikic BI, Letendre L, Dugan K, Lum B, Chin DL, Dewald G, Paietta E, Bennett JM, Rowe JM. Mitoxantrone, etoposide, and cytarabine with or without valspodar in patients with relapsed or refractory acute myeloid leukemia and high-risk myelodysplastic syndrome: a phase III trial (E2995). *J Clin Oncol* 2004; **22**: 1078-1086 [PMID: 15020609]

- DOI: 10.1200/jco.2004.07.048]
- 129 **Minderman H**, O'Loughlin KL, Pendyala L, Baer MR. VX-710 (biricodar) increases drug retention and enhances chemosensitivity in resistant cells overexpressing P-glycoprotein, multidrug resistance protein, and breast cancer resistance protein. *Clin Cancer Res* 2004; **10**: 1826-1834 [PMID: 15014037 DOI: 10.1158/1078-0432.CCR-0914-3]
 - 130 **Gottesman MM**, Ludwig J, Xia D, Szakács G. Defeating drug resistance in cancer. *Discov Med* 2006; **6**: 18-23 [PMID: 17234123]
 - 131 **Tang R**, Faussat AM, Perrot JY, Marjanovic Z, Cohen S, Storme T, Morjani H, Legrand O, Marie JP. Zosuquidar restores drug sensitivity in P-glycoprotein expressing acute myeloid leukemia (AML). *BMC Cancer* 2008; **8**: 51 [PMID: 18271955 DOI: 10.1186/1471-2407-8-51]
 - 132 **Kemper EM**, Cleypool C, Boogerd W, Beijnen JH, van Tellingen O. The influence of the P-glycoprotein inhibitor zosuquidar trihydrochloride (LY335979) on the brain penetration of paclitaxel in mice. *Cancer Chemother Pharmacol* 2004; **53**: 173-178 [PMID: 14605863 DOI: 10.1007/s00280-003-0720-y]
 - 133 **Dörner B**, Kuntner C, Bankstahl JP, Bankstahl M, Stanek J, Wanek T, Stundner G, Mairinger S, Löscher W, Müller M, Langer O, Erker T. Synthesis and small-animal positron emission tomography evaluation of [¹¹C]-elacridar as a radiotracer to assess the distribution of P-glycoprotein at the blood-brain barrier. *J Med Chem* 2009; **52**: 6073-6082 [PMID: 19711894 DOI: 10.1021/jm900940f]
 - 134 **Bates SE**, Shieh CY, Mickley LA, Dichek HL, Gazdar A, Loriaux DL, Fojo AT. Mitotane enhances cytotoxicity of chemotherapy in cell lines expressing a multidrug resistance gene (mdr-1/P-glycoprotein) which is also expressed by adrenocortical carcinomas. *J Clin Endocrinol Metab* 1991; **73**: 18-29 [PMID: 1675220 DOI: 10.1210/jcem-73-1-18]
 - 135 **Consoli U**, Priebe W, Ling YH, Mahadevia R, Griffin M, Zhao S, Perez-Soler R, Andreeff M. The novel anthracycline annamycin is not affected by P-glycoprotein-related multidrug resistance: comparison with idarubicin and doxorubicin in HL-60 leukemia cell lines. *Blood* 1996; **88**: 633-644 [PMID: 8695811]
 - 136 **Fox E**, Bates SE. Tariquidar (XR9576): a P-glycoprotein drug efflux pump inhibitor. *Expert Rev Anticancer Ther* 2007; **7**: 447-459 [PMID: 17428165 DOI: 10.1586/14737140.7.4.447]
 - 137 **Patel J**, Mitra AK. Strategies to overcome simultaneous P-glycoprotein mediated efflux and CYP3A4 mediated metabolism of drugs. *Pharmacogenomics* 2001; **2**: 401-415 [PMID: 11722289 DOI: 10.1517/14622416.2.4.401]
 - 138 **Fromm MF**. Importance of P-glycoprotein at blood-tissue barriers. *Trends Pharmacol Sci* 2004; **25**: 423-429 [PMID: 15276711 DOI: 10.1016/j.tips.2004.06.002]
 - 139 **van de Ven R**, Scheffer GL, Scheper RJ, de Groot TD. The ABC of dendritic cell development and function. *Trends Immunol* 2009; **30**: 421-429 [PMID: 19699682 DOI: 10.1016/j.it.2009.06.004]
 - 140 **Gekeler V**, Ise W, Sanders KH, Ulrich WR, Beck J. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. *Biochem Biophys Res Commun* 1995; **208**: 345-352 [PMID: 7887949 DOI: 10.1006/bbrc.1995.1344]
 - 141 **Kim HS**, Min YD, Choi CH. Double-edged sword of chemosensitizer: increase of multidrug resistance protein (MRP) in leukemic cells by an MRP inhibitor probenecid. *Biochem Biophys Res Commun* 2001; **283**: 64-71 [PMID: 11322768 DOI: 10.1006/bbrc.2001.4746]
 - 142 **O'Connor R**, O'Leary M, Ballot J, Collins CD, Kinsella P, Mager DE, Arnold RD, O'Driscoll L, Larkin A, Kennedy S, Fennelly D, Clynes M, Crown J. A phase I clinical and pharmacokinetic study of the multi-drug resistance protein-1 (MRP-1) inhibitor sulindac, in combination with epirubicin in patients with advanced cancer. *Cancer Chemother Pharmacol* 2007; **59**: 79-87 [PMID: 16642371 DOI: 10.1007/s00280-006-0240-7]
 - 143 **Rabindran SK**, Ross DD, Doyle LA, Yang W, Greenberger LM. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer Res* 2000; **60**: 47-50 [PMID: 10646850]
 - 144 **Tiwari AK**, Sodani K, Dai CL, Ashby CR Jr, Chen ZS. Revisiting the ABCs of multidrug resistance in cancer chemotherapy. *Curr Pharm Biotechnol* 2011; **12**: 570-594 [PMID: 21118094 DOI: 10.2174/138920111795164048]
 - 145 **Zhang YK**, Wang YJ, Gupta P, Chen ZS. Multidrug Resistance Proteins (MRPs) and Cancer Therapy. *AAPS J* 2015; **17**: 802-812 [PMID: 25840885 DOI: 10.1208/s12248-015-9757-1]
 - 146 **Zhou SF**, Wang LL, Di YM, Xue CC, Duan W, Li CG, Li Y. Substrates and inhibitors of human multidrug resistance associated proteins and the implications in drug development. *Curr Med Chem* 2008; **15**: 1981-2039 [PMID: 18691054 DOI: 10.2174/092986708785132870]
 - 147 **Widmer N**, Rumpold H, Untergasser G, Fayet A, Buclin T, Decosterd LA. Resistance reversal by RNAi silencing of MDR1 in CML cells associated with increase in imatinib intracellular levels. *Leukemia* 2007; **21**: 1561-1562; author reply 1562-1564 [PMID: 17429432 DOI: 10.1038/sj.leu.2404671]
 - 148 **Mechetner EB**, Roninson IB. Efficient inhibition of P-glycoprotein-mediated multidrug resistance with a monoclonal antibody. *Proc Natl Acad Sci USA* 1992; **89**: 5824-5828 [PMID: 1352877 DOI: 10.1073/pnas.89.13.5824]
 - 149 **Fisher M**, Abramov M, Van Aerschoot A, Xu D, Juliano RL, Herdewijn P. Inhibition of MDR1 expression with alitritol-modified siRNAs. *Nucleic Acids Res* 2007; **35**: 1064-1074 [PMID: 17264131 DOI: 10.1093/nar/gkl1126]
 - 150 **Wu H**, Hait WN, Yang JM. Small interfering RNA-induced suppression of MDR1 (P-glycoprotein) restores sensitivity to multidrug-resistant cancer cells. *Cancer Res* 2003; **63**: 1515-1519 [PMID: 12670898]
 - 151 **Wang Z**, Li Y, Ahmad A, Azmi AS, Kong D, Banerjee S, Sarkar FH. Targeting miRNAs involved in cancer stem cell and EMT regulation: An emerging concept in overcoming drug resistance. *Drug Resist Updat* 2010; **13**: 109-118 [PMID: 20692200 DOI: 10.1016/j.drug.2010.07.001]
 - 152 **Ma J**, Dong C, Ji C. MicroRNA and drug resistance. *Cancer Gene Ther* 2010; **17**: 523-531 [PMID: 20467450 DOI: 10.1038/cgt.2010.18]
 - 153 **Giovannetti E**, Funel N, Peters GJ, Del Chiaro M, Eroenci LA, Vasile E, Leon LG, Pollina LE, Groen A, Falcone A, Danesi R, Campani D, Verheul HM, Boggi U. MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. *Cancer Res* 2010; **70**: 4528-4538 [PMID: 20460539 DOI: 10.1158/0008-5472.can-09-4467]
 - 154 **Zhu H**, Wu H, Liu X, Evans BR, Medina DJ, Liu CG, Yang JM. Role of MicroRNA miR-27a and miR-451 in the regulation of MDR1/P-glycoprotein expression in human cancer cells. *Biochem Pharmacol* 2008; **76**: 582-588 [PMID: 18619946 DOI: 10.1016/j.bcp.2008.06.007]
 - 155 **Wang F**, Xue X, Wei J, An Y, Yao J, Cai H, Wu J, Dai C, Qian Z, Xu Z, Miao Y. hsa-miR-520h downregulates ABCG2 in pancreatic cancer cells to inhibit migration, invasion, and side populations. *Br J Cancer* 2010; **103**: 567-574 [PMID: 20628378 DOI: 10.1038/sj.bjc.6605724]
 - 156 **Hwang JH**, Voortman J, Giovannetti E, Steinberg SM, Leon LG, Kim YT, Funel N, Park JK, Kim MA, Kang GH, Kim SW, Del Chiaro M, Peters GJ, Giaccone G. Identification of microRNA-21 as a biomarker for chemoresistance and clinical outcome following adjuvant therapy in resectable pancreatic cancer. *PLoS One* 2010; **5**: e10630 [PMID: 20498843 DOI: 10.1371/journal.pone.0010630]
 - 157 **Ozvegy-Laczka C**, Cserepes J, Elkind NB, Sarkadi B. Tyrosine kinase inhibitor resistance in cancer: role of ABC multidrug transporters. *Drug Resist Updat* 2005; **8**: 15-26 [PMID: 15939339 DOI: 10.1016/j.drug.2005.02.002]
 - 158 **Brendel C**, Scharenberg C, Dohse M, Robey RW, Bates SE, Shukla S, Ambudkar SV, Wang Y, Wennemuth G, Burchert A, Boudriot U, Neubauer A. Imatinib mesylate and nilotinib (AMN107) exhibit high-affinity interaction with ABCG2 on primitive hematopoietic

- stem cells. *Leukemia* 2007; **21**: 1267-1275 [PMID: 17519960 DOI: 10.1038/sj.leu.2404638]
- 159 **Dai CL**, Liang YJ, Wang YS, Tiwari AK, Yan YY, Wang F, Chen ZS, Tong XZ, Fu LW. Sensitization of ABCG2-overexpressing cells to conventional chemotherapeutic agent by sunitinib was associated with inhibiting the function of ABCG2. *Cancer Lett* 2009; **279**: 74-83 [PMID: 19232821 DOI: 10.1016/j.canlet.2009.01.027]
- 160 **Dai CL**, Tiwari AK, Wu CP, Su XD, Wang SR, Liu DG, Ashby CR Jr, Huang Y, Robey RW, Liang YJ, Chen LM, Shi CJ, Ambudkar SV, Chen ZS, Fu LW. Lapatinib (Tykerb, GW572016) reverses multidrug resistance in cancer cells by inhibiting the activity of ATP-binding cassette subfamily B member 1 and G member 2. *Cancer Res* 2008; **68**: 7905-7914 [PMID: 18829547 DOI: 10.1158/0008-5472.can-08-0499]
- 161 **Kuang YH**, Shen T, Chen X, Sodani K, Hopper-Borge E, Tiwari AK, Lee JW, Fu LW, Chen ZS. Lapatinib and erlotinib are potent reversal agents for MRP7 (ABCC10)-mediated multidrug resistance. *Biochem Pharmacol* 2010; **79**: 154-161 [PMID: 19720054 DOI: 10.1016/j.bcp.2009.08.021]
- 162 **Livney YD**, Assaraf YG. Rationally designed nanovehicles to overcome cancer chemoresistance. *Adv Drug Deliv Rev* 2013; **65**: 1716-1730 [PMID: 23954781 DOI: 10.1016/j.addr.2013.08.006]
- 163 **Sun T**, Zhang YS, Pang B, Hyun DC, Yang M, Xia Y. Engineered nanoparticles for drug delivery in cancer therapy. *Angew Chem Int Ed Engl* 2014; **53**: 12320-12364 [PMID: 25294565 DOI: 10.1002/anie.201403036]
- 164 **Xue X**, Liang XJ. Overcoming drug efflux-based multidrug resistance in cancer with nanotechnology. *Chin J Cancer* 2012; **31**: 100-109 [PMID: 22237039 DOI: 10.5732/cjc.011.10326]
- 165 **Kool M**, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F, Borst P. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res* 1997; **57**: 3537-3547 [PMID: 9270026]
- 166 **Kaye SB**. Reversal of drug resistance in ovarian cancer: where do we go from here? *J Clin Oncol* 2008; **26**: 2616-2618 [PMID: 18509172 DOI: 10.1200/jco.2008.16.2123]
- 167 **Relling MV**. Are the major effects of P-glycoprotein modulators due to altered pharmacokinetics of anticancer drugs? *Ther Drug Monit* 1996; **18**: 350-356 [PMID: 8857549]
- 168 **Ozols RF**, Cunnion RE, Klecker RW Jr, Hamilton TC, Ostchega Y, Parrillo JE, Young RC. Verapamil and adriamycin in the treatment of drug-resistant ovarian cancer patients. *J Clin Oncol* 1987; **5**: 641-647 [PMID: 3559654 DOI: 10.1200/jco.1987.5.4.641]
- 169 **Park S**, Shimizu C, Shimoyama T, Takeda M, Ando M, Kohno T, Katsumata N, Kang YK, Nishio K, Fujiwara Y. Gene expression profiling of ATP-binding cassette (ABC) transporters as a predictor of the pathologic response to neoadjuvant chemotherapy in breast cancer patients. *Breast Cancer Res Treat* 2006; **99**: 9-17 [PMID: 16752223 DOI: 10.1007/s10549-006-9175-2]
- 170 **Marzac C**, Garrido E, Tang R, Fava F, Hirsch P, De Benedictis C, Corre E, Lapusan S, Lallemand JY, Marie JP, Jacquet E, Legrand O. ATP Binding Cassette transporters associated with chemoresistance: transcriptional profiling in extreme cohorts and their prognostic impact in a cohort of 281 acute myeloid leukemia patients. *Haematologica* 2011; **96**: 1293-1301 [PMID: 21606172 DOI: 10.3324/haematol.2010.031823]
- 171 **Chen Z**, Shi T, Zhang L, Zhu P, Deng M, Huang C, Hu T, Jiang L, Li J. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family in multidrug resistance: A review of the past decade. *Cancer Lett* 2016; **370**: 153-164 [PMID: 26499806 DOI: 10.1016/j.canlet.2015.10.010]
- 172 **Kim RB**. Drugs as P-glycoprotein substrates, inhibitors, and inducers. *Drug Metab Rev* 2002; **34**: 47-54 [PMID: 11996011 DOI: 10.1081/dmr-120001389]
- 173 **Slot AJ**, Molinski SV, Cole SP. Mammalian multidrug-resistance proteins (MRPs). *Essays Biochem* 2011; **50**: 179-207 [PMID: 21967058 DOI: 10.1042/bse0500179]

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Rethinking *de novo* immune hepatitis, an old concept for liver allograft rejection: Relevance of glutathione S-transferase T1 mismatch

Isabel Aguilera, Elena Aguado-Dominguez, Jose Manuel Sousa, Antonio Nuñez-Roldan

Isabel Aguilera, Elena Aguado-Dominguez, Antonio Nuñez-Roldan, Department of Immunology, Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío, Sevilla 41013, Spain

Jose Manuel Sousa, Digestive and Liver Diseases Service, Hospital Universitario Virgen del Rocío, Sevilla 41013, Spain

ORCID number: Isabel Aguilera (0000-0003-2165-4954); Elena Aguado-Dominguez (0000-0003-1519-6002); Jose Manuel Sousa (0000-0002-8158-273X); Antonio Nuñez-Roldan (0000-0001-6479-2586).

Author contributions: Aguilera I contributed to this paper with the conception, literature review and drafting; Aguado-Dominguez E contributed with literature review, original data and drafting; Sousa JM and Nuñez-Roldan A contributed with critical revision of the manuscript and approval of the final version.

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Correspondence to: Isabel Aguilera, PhD, Research Fellow, Senior Scientist, Department of Immunology, Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío, Avda Manuel Siurot s/n, Sevilla 41013, Spain. iaguilera-ibis@us.es

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Abstract

Antibody-mediated rejection (AMR) in liver transplantation has long been underestimated. The concept of the liver as an organ susceptible to AMR has emerged in recent years, not only in the context of the major histocompatibility complex with the presence of HLA donor-specific antibodies, but also with antigens regarded as "minor", whose role in AMR has been demonstrated. Among them, antibodies against glutathione S-transferase T1 have been found in 100% of patients with *de novo* autoimmune hepatitis (dnAIH) when studied. In its latest update, the Banff Working Group for liver allograft pathology proposed replacing the term dnAIH with plasma cell (PC)-rich rejection. Antibodies to glutathione S-transferase T1 (GSTT1) in null recipients of GSTT1 positive donors have been included as a contributory but nonessential feature of the diagnosis of PC-rich rejection. Also in this update, non-organ-specific anti-nuclear or smooth muscle autoantibodies are no longer included as diagnostic criteria. Although initially found in a proportion of patients with PC-rich rejection, the presence of autoantibodies is misleading since they are not disease-specific and appear in many different contexts as bystanders. The cellular types and proportions of the inflammatory infiltrates in diagnostic biopsies have been studied in detail very recently. PC-rich rejection

biopsies present a characteristic cellular profile with a predominance of T lymphocytes and a high proportion of PCs, close to 30%, of which 16.48% are IgG4⁺. New data on the relevance of GSTT1-specific T lymphocytes to PC-rich rejection will be discussed in this review.

Key words: Glutathione S-transferase T1 mismatch; Liver allograft rejection; Plasma cell-rich rejection; *De novo* autoimmune hepatitis; Donor-specific antibodies; NewCAST; Cell quantification; IgG4⁺ plasma cell; T lymphocytes

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Core tip: The purpose of this review is to update the reader with recent knowledge about a disease of the liver allograft, whose definition has evolved from “*de novo* autoimmune hepatitis” to “plasma cell-rich rejection”. During the last 20 years, several groups have contributed new data that has prompted the liver transplant community to reconsider several aspects of the disease. It is not the intention of this review to go over details of the histological features or the role of autoantibodies in this disease, which have been well described in other reviews. Instead, more recent aspects, such as the composition of infiltrates in biopsies and T cell involvement will be discussed.

Aguilera I, Aguado-Dominguez E, Sousa JM, Nuñez-Roldan A. Rethinking *de novo* immune hepatitis, an old concept for liver allograft rejection: Relevance of glutathione S-transferase T1 mismatch. *World J Gastroenterol* 2018; 24(29): 3239-3249 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i29/3239.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i29.3239>

INTRODUCTION

Antibody-mediated rejection (AMR) in liver transplantation is becoming increasingly relevant after being considered an immune privileged organ for many years. Indeed, a good HLA match between donor and recipient - very important in other settings such as kidney transplants - was never considered as essential in liver donations. A few years ago, a number of publications describing a pathogenic role for HLA donor-specific antibodies (DSA) came out indicating that the liver was prone to experience AMR like any other organ^[1]. Earlier, in 1998, a new liver transplant-associated disease termed *de novo* autoimmune hepatitis (dnAIH) was described^[2] and many groups reported cases of patients with similar characteristics but with different prevalence^[3-25]. The diagnostic criteria are well described, particularly with regard to histological features that are essential for differential diagnosis in adult^[26-32] and pediatric^[9,33] cases. To complete the characterization of this special type of immune response, now generally accepted as rejection

but with disconcerting similarities with autoimmunity^[34], there are a few aspects that still need to be investigated.

For example, one important issue that has yet to be addressed is the role of allelic disparity of glutathione S-transferase T1 (GSTT1) or other minor histocompatibility antigen mismatches in the development of dnAIH in pediatric liver transplant. There are very few studies about the long-term consequences of dnAIH in the liver allograft of children. Ekong *et al*^[14] reported their observations from a retrospective multi-center study that included 29 children from 5 centers. The authors showed that half of the patients did not experience rejection prior to diagnosis and the response to steroid therapy was good in general but not in all the cases. Interestingly, 38% of the children had abnormal liver enzymes over 2-fold the upper limit of normal, especially gamma-glutamyltransferase (GGT) at the time of last follow-up, indicating bile duct injury. This result contradicts one of the main arguments against considering dnAIH a type of rejection, namely the absence of bile duct involvement.

Well-established immunological criteria for diagnosing AMR in kidney transplantation include detection of complement component 4d (C4d) deposits in peritubular capillaries concomitantly with antidonor serology^[35]. Presently, C4d deposition in portal capillaries is accepted as a distinctive feature of dnAIH/PC hepatitis^[36,37] although it is not currently considered to be a diagnostic criterion.

IgG4 has been traditionally considered a benign antibody although this concept has changed due to the growing number of IgG4-related diseases described in the literature during the last few years. International experts in the field held a symposium in Boston in 2012 and generated consensus guidelines for the diagnosis of IgG4-related diseases^[38]. Since an important presence of IgG4⁺ plasma cells (PCs) has been detected in subgroups of patients with dnAIH/PC-rich rejection, this aspect will be discussed later in this review.

TERMINOLOGY CONTROVERSY

When pathologists first identified the transplant-associated pathology dnAIH, the histological features described were very similar to those found in autoimmune hepatitis^[2]. Following this first study in 1998^[2], dnAIH became the term of choice^[6-8,11,12,17,22,25,39,40]. However, from the beginning, clinicians found it difficult to admit progression to autoimmunity in a patient's graft without a previous history of autoimmune phenomena and under the effects of immunosuppressive therapy. Moreover, the graft targeted by the immune reaction was not self but coming from a donor with a completely different genetic background, given the fact that selection was very uncommon, even for the HLA antigens. Soon after the first description, some authors started using other terms such as “*de novo* hepatitis”^[43], “graft dysfunction mimicking autoimmune hepatitis”^[16], “posttransplant immune hepatitis”^[44] or *de novo* immune

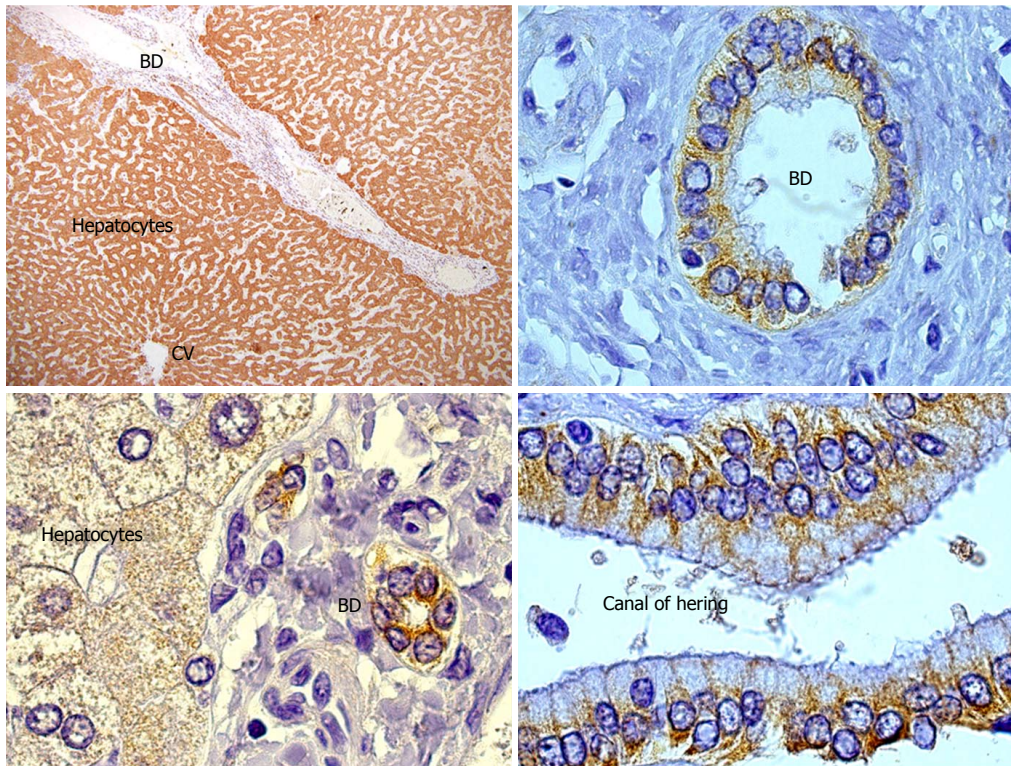


Figure 1 *GSTT1* expression in human liver. On the left part of the figure the hepatic parenchyma shows homogeneous staining of the cytoplasm of hepatocytes as well as epithelial cells of the bile ducts in the portal area. On the right part, a detail of the cytoplasmic staining of cholangiocytes and the Canal of Hering cells, considered a niche of hepatic progenitor cells. CV: Central vein; BD: Bile duct.

hepatitis^[18]. In 2008, a study performed by Fiel *et al.*^[23] introduced the term “plasma cell hepatitis” with the suggestion that dnAIH could be a variant of rejection; this report was highlighted by an editorial comment^[34]. Even though all these terms have been used historically, for simplicity, in this review we will refer to dnAIH or PC-rich rejection.

GLUTATHIONE S-TRANSFERASE T1: A MINOR HISTOCOMPATIBILITY ANTIGEN

GSTT1 is a phase II drug metabolizing enzyme involved in protection against toxic compounds. Vital for this function is its abundant expression in liver and kidney (Figure 1), although GSTT1 is also expressed in other tissues to a lesser degree. Importantly, the GSTT1 antigen is present in red blood cells. It has been demonstrated that any healthy person with the *GSTT1**0/0 genotype might produce anti-GSTT1 antibodies if they experience one of two sensitization events: blood transfusions from a GSTT1-positive donor or pregnancy of a GSTT1-positive fetus^[41]. Mismatch of the GSTT1 alleles was first reported by Aguilera *et al.*^[42] and subsequently confirmed in patients with PC-rich rejection^[18,21,24,43]. Czaja included GSTT1 antibodies as a feature in dnAIH patients and proposed a hypothesis that will be discussed in the PATHOGENESIS OF PC-RICH REJECTION section of this manuscript^[44]. Controversially, one report did not find GSTT1 mismatch

in a patient with dnAIH; however, the genotyping results were not very convincing since they lacked an internal control band and GSTT1 antibodies were not tested^[22]. To the best of our knowledge, anti-GSTT1 antibodies have never been tested in serum samples from liver transplanted children.

We studied the cases of two female patients with preformed anti-GSTT1 antibodies who needed a liver transplant and received a GSTT1 expressing graft. Their original diseases were primary and secondary biliary cirrhosis, respectively. The first patient presented high titers of GSTT1 antibodies and was diagnosed with dnAIH 12 mo after the transplant with a good response to prednisone plus mycophenolate mofetil. She died of pneumonia 4 years after the transplant. The second patient had low antibody titers in different samples until exitus, 7 years after the transplant. This patient was never diagnosed correctly and the only biopsy, performed one month before exitus, revealed dnAIH as the cause of exitus. Since these pre-sensitized patients are not frequent, it is difficult to conclude whether the presence of preformed antibodies accelerates the onset or the severity of the disease.

IN FAVOR OF ANTIBODY-MEDIATED REJECTION IN *DE NOVO* AIH

At the beginning of this decade a report by Aguilera *et al.*^[36], that was worth an editorial comment^[45] was

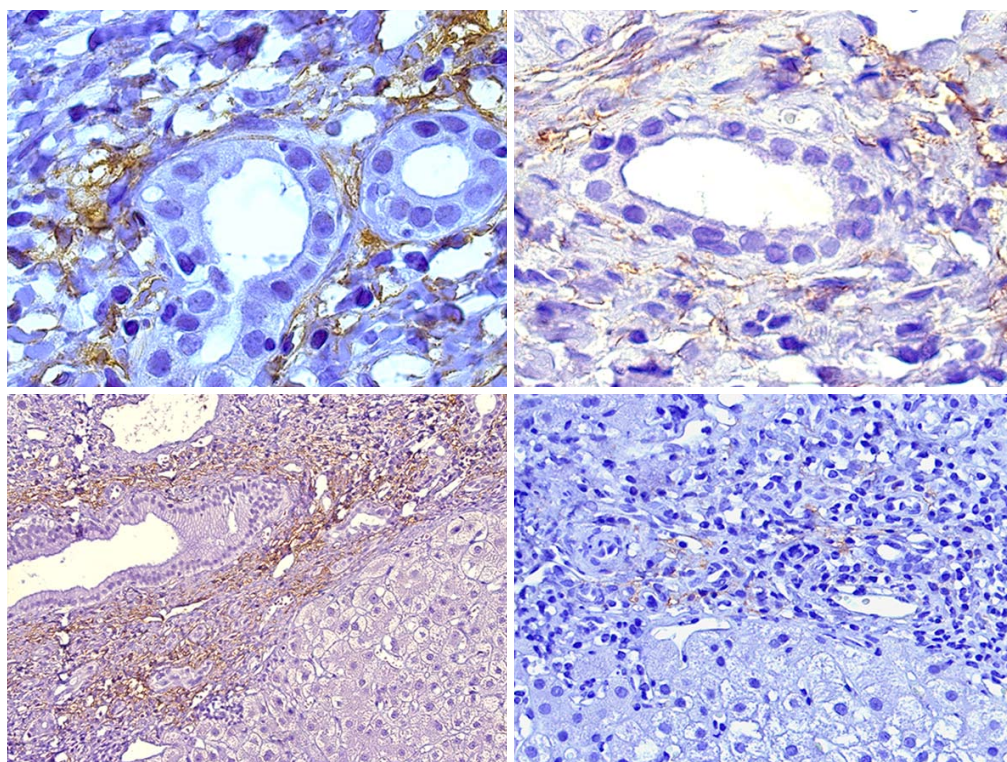


Figure 2 Portal areas in diagnostic biopsies of patients with plasma cell-rich rejection are shown. Staining of C4d deposits is observed in portal capillaries indicating antibody-mediated rejection.

the first to link C4d immunopositivity in liver biopsies to donor-specific alloreactivity not related to HLA DSA or ABO incompatibility^[46]. Patients with anti-GSTT1 DSA showed a very characteristic C4d immunostaining pattern restricted to portal capillaries (Figure 2), very distinct to the sinusoidal pattern described by Kozłowski *et al.*^[47] in association with HLA DSA, which was similar to our own findings in chronic rejection biopsies^[36].

Dumortier *et al.*^[48] described a case of refractory dnAIH that did not respond to standard therapy in a patient with anti-LKM (liver-kidney microsomal) antibodies at the time of diagnosis. Recovery after treatment with plasmapheresis supported an involvement of humoral factors in the pathogenesis of the disease.

In the last publication by the Banff Working Group for liver allograft pathology, the proposed criteria included HLA DSA and GSTT1 in null recipients of GSTT1⁺ positive donors as contributory but nonessential features for the diagnosis of PC-rich rejection^[1].

HLA DQ DSAs have been described as a predictive variable for late allograft dysfunction in children^[49]. Unfortunately, the number of patients in this study with dnAIH ($n = 3$) was too low to reach a conclusion and, even with the inclusion of 10 more patients with overlapping features of dnAIH and late ACR, the results should be considered carefully since it is not possible to analyze both events separately. We studied HLA DSA in a group of adult patients with dnAIH and found that 4 out of 14 (28.6%) produced *de novo* HLA DQ DSA, but always coexisting with GSTT1 DSAs (unpublished

results), making it impossible to differentiate the real impact of HLA DSA on the development of PC-rich rejection. It would be very interesting to validate these results in larger cohorts in order to determine the pathogenic role of HLA DSA in the absence of anti-GSTT1 antibodies.

THE THIN LINE BETWEEN AUTO- AND ALLO-IMMUNITY

Although finally accepted as a form of allograft rejection, the liver transplant-associated disease formerly known as dnAIH is a special kind of rejection and one that is difficult to understand. It has many histological similarities with classical AIH, features that were misleading at the time this pathology was discovered. Moreover, the first-line therapy of choice is corticosteroids for both pathological processes. All of this supports the concept that an alloantigenic immune response may be difficult to distinguish from an autoimmune response in which the mechanisms of liver damage are probably similar. Interestingly, we noted a female predominance among patients who present with one autoimmune feature, since of the cases of dnAIH diagnosed at our center, 10 of 14 are female (71.4%), a feature that is not easy to explain if we contemplate that 75% of the liver transplanted patients in our hospital are males. Moreover, if we consider the 122 patients within the risk group with GSTT1 mismatch (positive donor/null recipient), 85 were males (69.7%) and 37 were females (30.3%). In

Table 1 Reported cases of de novo autoimmune hepatitis in pediatric and adult liver transplantation

Ref.	de novo AIH/total patients	Original disease (immune-mediated)	Initial calcineurine inhibitor	Pediatric/adult
Kerkar <i>et al</i> ^[2] (1998)	7/180	4 BA	4 CyA, 3 Tac	P
Gupta <i>et al</i> ^[3] (2001)	6/115	5 BA	6 CyA	P
Andries <i>et al</i> ^[4] (2001)	11/471	7 BA	10 CyA, 1 Tac	P
Hernández <i>et al</i> ^[5] (2001)	5/155	4 BA, 1 PSC	5 CyA	P
Petz <i>et al</i> ^[6] (2002)	18/155	16 BA	9 CyA, 9 Tac	P
Miyagawa-Hayashino <i>et al</i> ^[7] (2003)	1	BA	Tac	P
Gibelli <i>et al</i> ^[8] (2006)	2/206	1 BA	CyA	P
Evans <i>et al</i> ^[9] (2006)	4/158	4 PSC	4 CyA	P
Riva <i>et al</i> ^[10] (2006)	9/247	9 BA	5 CyA, 4 Tac	P
Oya <i>et al</i> ^[11] (2009)	1	-	Tac	P
Cho <i>et al</i> ^[12] (2011)	4/148	1 BA	-	P
Pongpaibul <i>et al</i> ^[13] (2012)	51/685	29 BA	22 CyA, 29 Tac	P
Ekong <i>et al</i> ^[14] (2017)	29/1833	17 BA	13 CyA, 16 Tac	P
Jones <i>et al</i> ^[15] (1999)	2	2 PBC	2 CyA	A
Heneghan <i>et al</i> ^[16] (2001)	7/1000	1 PBC, 2 PSC	7 CyA	A
Salcedo <i>et al</i> ^[17] (2002)	12/350	1 BA 1 PSC	12 CyA	A
Aguilera <i>et al</i> ^[18] (2004)	6/110	None	5 CyA, 1 Tac	A
¹ Update 2017	8	1 PBC, 1 PSC	8 CyA	A
Miyagawa-Hayashino <i>et al</i> ^[19] (2004)	13/633	11 BA	13 Tac	A
Tsuji <i>et al</i> ^[20] (2005)	1	PBC	CyA	A
Rodriguez-Diaz <i>et al</i> ^[21] (2006)	1	PBC	CyA	A
Yoshizawa <i>et al</i> ^[22] (2008)	1	PBC	Tac	A
Fiel <i>et al</i> ^[23] (2008)	38/?	none (HCV)	11 CyA, 27 Tac	A
Zhang <i>et al</i> ^[24] (2010)	1	PBC	Tac	A
Montano-Loza <i>et al</i> ^[25] (2012)	17/576	5 PBC, 1 BA	5 CyA, 7 Tac	A

¹Presently we have 8 more patients diagnosed. BA: Biliary atresia; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis; CyA: Cyclosporine A; Tac: Tacrolimus; A: Adult; P: Pediatric.

accordance with female predominance are the reports by Miyagawa-Hayashino^[7,19], in which 12 of 14 cases were females, and Pongpaibul *et al*^[13] with 32 females vs 19 males diagnosed with dnAIH, although in the majority of publications this predominance does not appear. Ward *et al*^[50] established that women may be more susceptible to PC hepatitis after liver transplant than men. A reasonable explanation could be sensitization through pregnancies. In fact, we have two female patients with GSTT1 antibodies in pre-transplant serum samples, although we do not have a sufficient number of cases to support this hypothesis.

AUTOIMMUNE OR IMMUNE MEDIATED PRETRANSPLANT PATHOLOGIES: HAVE THEY ANY INFLUENCE IN PC-RICH REJECTION?

It is generally accepted that dnAIH develops in patients transplanted for non-autoimmune diseases. This is not exactly true since there are a number of patients whose original disease was either primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC) (Table 1). Neuberger's group found that PBC was an independent predictor of late acute rejection in a large cohort of adult liver transplant patients^[51].

In children, the major indication for liver transplant is

biliary atresia (BA), a process of unknown etiology with several potential causative factors. Among them, there is evidence in favor of autoimmune-mediated injury of bile duct epithelial cells in a subset of patients^[52-54]. Very recently, a study described that BA is initiated before birth and the presence of maternal microchimerism in the BA liver supports graft versus host disease (GvHD)-like immune response^[55].

T CELL INVOLVEMENT IN PC-RICH REJECTION

A possible role of GSTT1-specific T lymphocytes in PC-rich rejection has been recently investigated. The authors provided the first evidence of memory specific T cells in samples of peripheral blood mononuclear cells (PBMC) of patients after recall with the GSTT1 antigen. Activation of CD8⁺ and CD4⁺ T cells with production of IL-4 and/or IFN γ was observed^[56]. Most intriguing was the finding of highly activated CD4⁺ cells that retained CD8^{low} expression with a mean value of 25% activated Th0 type cells (3.44%-78.95%). These results are particularly significant considering the immunosuppressed status of the patients. In silico analysis of the ability of patients' HLA class I and class II alleles to present these peptides with optimal percentile ranks supported the experimental results^[56].

Similar to autoimmune hepatitis, we observe damage

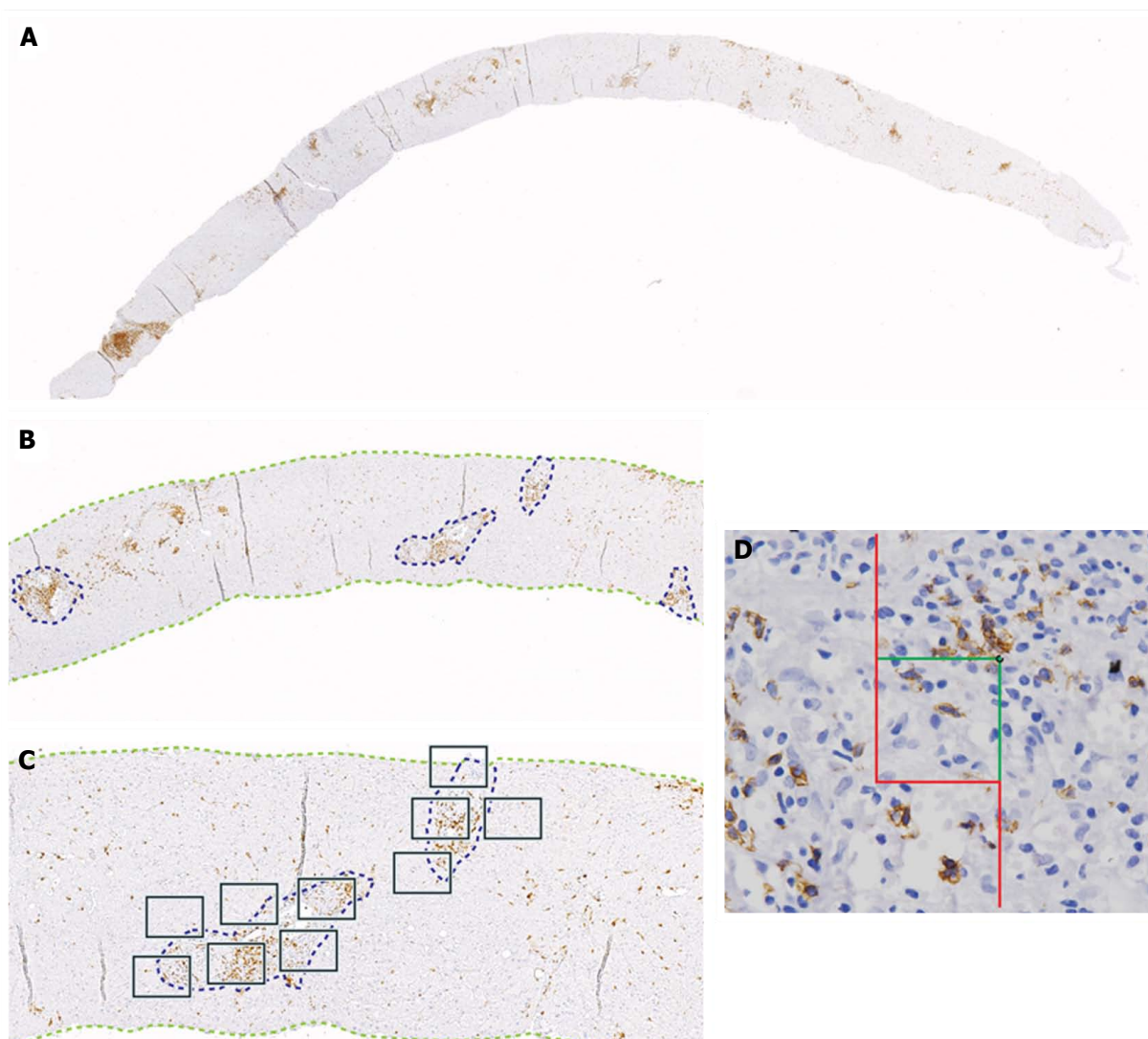


Figure 3 Representative images showing quantification procedure using NewCAST™ Visiopharm® software. A: Super image capture of a slide; B: Computer-assisted drawing of the tissue (green) and manually drawing of the regions of interest (ROI) or portal areas (blue); C: Example of the unbiased sampling; D: A detail of cell quantification inside the counting frame.

to hepatocytes that is believed to be coordinated by CD4⁺ T lymphocytes recognizing a self-antigen presented by HLA-class II molecules to Th0 type cells^[57], although with the substantial difference that, in this case, the target is an alloantigen.

WHICH CELLS ARE INFILTRATING THE LIVER AT THE ONSET OF PC-RICH REJECTION?

Diagnosis by liver biopsy is challenging because PC-rich rejection shares some histological and clinical features with late onset acute rejection or with other post-transplant pathologies such as recurrent hepatitis C. Moreover, very little is known about the cellular composition of the inflammatory infiltrates in portal tracts other than an important presence of plasma cells. Direct visualization of the cell types with different

markers and quantification of cells/mm² of tissue in diagnostic biopsies using computer-assisted system technology (newCAST) has proved to be an unbiased way to evaluate the situation directly in the organ and not only in peripheral blood (Figure 3). Very recent data allowed us to define a cellular profile characteristic of PC-rich rejection at the time of diagnosis^[58]. We observed a predominance of T lymphocytes (mean 36.6%); followed by PCs (mean 28.8%), with 17% of them IgG4⁺; B cells (mean 14.9%); and macrophages (mean 19.7%) (Figure 4). This profile was very different to the one defined in a chronic rejection group included in the same study^[58]. It remains to be confirmed whether the cellular composition in acute rejection biopsies would be useful to support diagnosis since HCV recurrence no longer has the same impact in the transplant community that as in the past.

The IgG4 subclass has traditionally been considered a modulator of the immune response, linked to long-

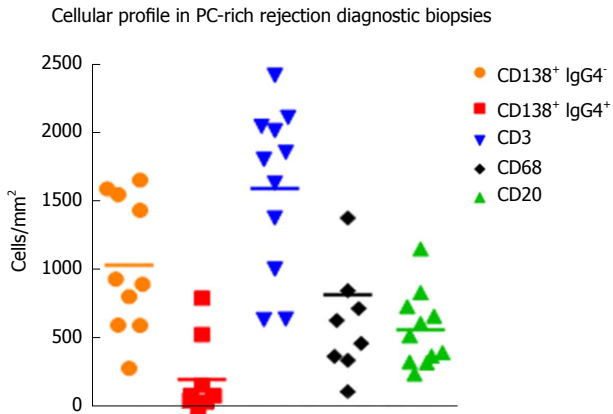


Figure 4 Total cell count/ area of tissue in diagnostic biopsies with the following markers: CD138⁺ plasma cells, IgG4⁺ plasma cells, CD3⁺ T lymphocytes, CD68⁺ macrophages, CD20⁺ B lymphocytes. Mean values are shown.

term exposure and high antigen concentration. Some authors analyzed the presence of IgG4 antibodies in dnAIH, mostly in children, but the results were contradictory. While Eguchi *et al.*^[59] described the absence of IgG4⁺ plasma cells in the biopsies of 4 patients and the values in serum were within normal limits, Castillo-Rama *et al.*^[60] detected IgG4⁺ PC over-representation in a subgroup of patients with PC hepatitis that showed a more severe disease course. The origin of these IgG4⁺ PC infiltrates in the allografts is unknown but it could be due to persistent alloimmune responses. We also found an important number of IgG4⁺ PC, representing 16.48% (2.45%-65%) of the total PCs in diagnostic biopsies of patients with PC-rich rejection (Figure 5A and B); however, higher numbers of IgG4⁺ cells was not associated with worse clinical outcome. We also found an over-representation of serum levels of GSTT1-specific IgG4 antibodies, almost equaling the levels of GSTT1-IgG1, while GSTT1-IgG2 and -IgG3 were completely absent (Figure 5C)^[61].

There is very little information about the representation of T cells in PC-rich rejection biopsies. A study performed by Ekong *et al.*^[62] found that around 3000 CD3⁺ cells/mm² were present in the dnAIH pediatric group during disease activity. Our data in adults showed that CD3⁺ cells were less numerous, with a mean value of 1600 (641-2422) cells/mm² at the time of diagnosis, before steroid treatment. Indirect evidence from biopsies suggests a potential role for Th17 cells in the prolongation of inflammation in dnAIH^[63] although this needs further study.

PC-RICH REJECTION IN HEMATOPOIETIC CELL TRANSPLANTATION

PC-rich rejection has also been identified after hematopoietic cell transplantation (HCT) between HLA-identical siblings. In this context, the GSTT1 mismatch, defined as null donor/positive recipient, not only had a

deleterious effect in HCT and constituted a risk factor for acute and chronic hepatic GvHD^[64] but is also the basis of a true PC-rich rejection^[65]. Together with the skin and intestine, the liver is a preferred target of the donor cells which are able to recognize disparate antigens expressed by the recipient. Differential diagnosis of hepatic GvHD versus PC-rich rejection must be done in biopsy because histological features can only be analyzed in tissue to permit the differentiation of both processes. As it happens, the cellular composition of the inflammatory infiltrates in portal areas of HCT patient with PC-rich rejection was very similar to that of diagnostic biopsies of liver transplanted patients^[58].

From an immunological point of view, this finding has important implications because it demonstrates an identical immune response against the GSTT1 antigen as a result of donor/recipient mismatch in two completely different conditions: Solid organ and HCT.

PATHOGENESIS OF PC-RICH REJECTION

It is clear that during surgery, intracellular antigens such as the GSTT1 protein are released and recognized by B cells, starting a process of affinity maturation and differentiation into memory cells, which requires collaboration with specific T helper cells in the lymph nodes. If this occurs, a subset of these cells will progress to plasma cells and anti-GSTT1 antibodies of the IgG class will be detected concurrently with mature GSTT1-specific CD4⁺ cells. Our experimental results sustain indirect presentation of the donor GSTT1 protein by recipient APCs^[56].

In general terms, we could use the model proposed by Czaja^[44] with some suggestions. Briefly, inflammatory stimuli induce expression of MHC class II in hepatocytes and cholangiocytes and, since both cellular types contain abundant GSTT1 enzyme, these liver cells could directly act as APCs and serve as targets of effector CD4⁺ T cells, whose existence has been also demonstrated in the PBMC of patients with the disease. The presence of an antigen mimicking GSTT1 on the surface of the cell cannot be ruled out but it does not seem necessary and so far has not been identified.

GSTT1-specific B cells have a critical role as antigen presenting cells (APCs) in the maintenance of a long-lasting immunological response, providing the signals required for specific T cell activation when professional APCs become exhausted. GSTT1 antibodies are, in principle and until a direct pathogenic role can be demonstrated, a signal of the existence of memory B cells. The mechanisms controlling GSTT1-specific T cell response in patients that, in spite of the production of GSTT1 antibodies, do not develop the disease are still not known. Alternatively, there are patients with GSTT1-specific T cells that lack the B cell response; they will never develop PC-rich rejection. In our experience, if PC-rich rejection is not initiated during the first 3 years, it will never occur even though anti-

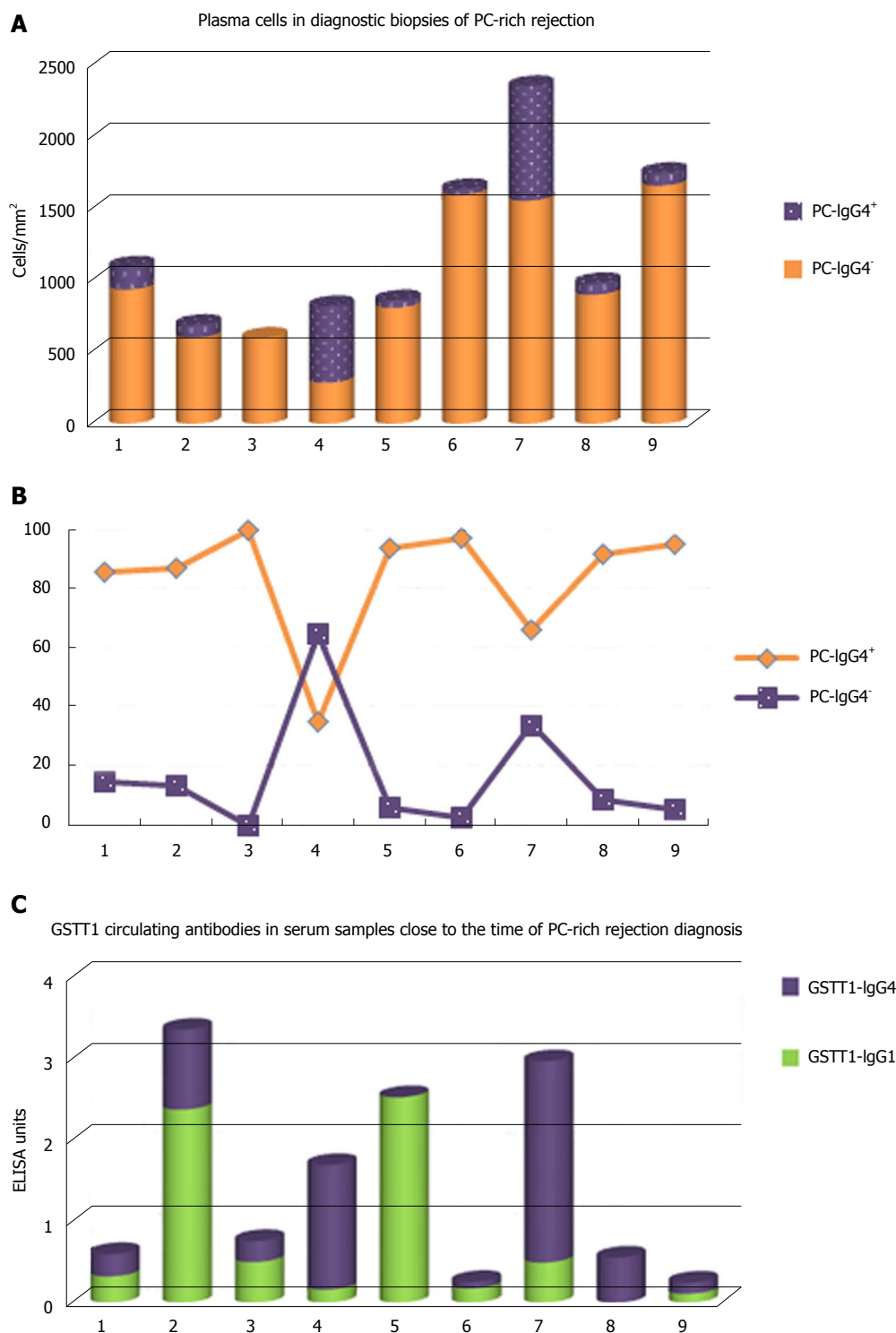


Figure 5 CD138⁺ plasma cells were quantified in the diagnostic biopsies of the 7 patients (A). The results are shown as number of cells per mm²/tissue. IgG4⁺ plasma cells were also counted and subtracted from the total number of CD138⁺ cells. B: The same results are represented as percentages. C: Level of anti-GSTT1 antibodies in serum samples close to the diagnostic biopsy of 7 patients with dnAIH; note that only IgG1 and IgG4 were present whereas IgG2 and IgG3 were absent. It is important to highlight that these are donor-specific antibodies.

GSTT1 antibodies persist (longest follow-up > 20 years) (unpublished results).

Impairment of Treg cells has been described in patients with classical AIH^[57]. In the transplant setting, a similar role for Tregs in the development of dnAIH has been suggested. Kerkar and Yanni proposed that Treg function could be impaired in dnAIH since calcineurin

inhibitors reduce the production of IL-2, which is required for the survival and proliferation of Tregs^[66]. In the same sense, a study by Arterbery *et al.*^[67] sustains that the Tregs of patients with dnAIH are functionally impaired and produce increased levels of proinflammatory cytokines.

On the other hand, important questions about dnAIH

have yet to be answered. One of the most intriguing aspects is why some patients do not develop a GSTT1-specific B cell response, as this is going to be critical to the prevention of PC-rich rejection. In our hands, the choice of tacrolimus instead of cyclosporine can be decisive in avoiding the humoral response^[68]. However, there is no consensus among the scientific community on this matter. While some studies support the observation of tacrolimus as a protective factor^[3-5,8,9,15-17,20,21], others have shown that the use of tacrolimus seems to be a risk factor for dnAIH^[13,14,19,22-25]; therefore, this point remains controversial (Table 1).

CONCLUSION

We are now closer than ever to clarification of the mechanisms leading to plasma cell-rich rejection. The demonstration of memory T cells specific for the GSTT1 antigen as well as B lymphocytes and GSTT1 antibody-producing plasma cells after GSTT1-mismatched liver transplants support the hypothesis in which both cell types are required to develop the immune response leading to PC-rich rejection. This chronic disease has a significant impact on survival of those patients that are not correctly diagnosed.

REFERENCES

- Demetris AJ, Bellamy C, Hübscher SG, O'Leary J, Randhawa PS, Feng S, Neil D, Colvin RB, McCaughan G, Fung JJ, Del Bello A, Reinholt FP, Haga H, Adeyi O, Czaja AJ, Schiano T, Fiel MI, Smith ML, Sebach M, Tanigawa RY, Yilmaz F, Alexander G, Baiocchi L, Balasubramanian M, Batal I, Bhan AK, Bucuvalas J, Cerski CTS, Charlotte F, de Vera ME, ElMonayeri M, Fontes P, Furth EE, Gouw ASH, Hafezi-Bakhtiari S, Hart J, Honsova E, Ismail W, Itoh T, Jhala NC, Khettry U, Klintmalm GB, Knechtle S, Koshiha T, Kozlowski T, Lassman CR, Lerut J, Levitsky J, Licini L, Liotta R, Mazariegos G, Minervini MI, Misdraji J, Mohanakumar T, Mölne J, Nasser I, Neuberger J, O'Neil M, Pappo O, Petrovic L, Ruiz P, Sağol Ö, Sanchez Fueyo A, Sasatomi E, Shaked A, Shiller M, Shimizu T, Sis B, Sonzogni A, Stevenson HL, Thung SN, Tisone G, Tsamandas AC, Wernerson A, Wu T, Zeevi A, Zen Y. 2016 Comprehensive Update of the Banff Working Group on Liver Allograft Pathology: Introduction of Antibody-Mediated Rejection. *Am J Transplant* 2016; **16**: 2816-2835 [PMID: 27273869 DOI: 10.1111/ajt.13909]
- Kerker N, Hadzić N, Davies ET, Portmann B, Donaldson PT, Rela M, Heaton ND, Vergani D, Mieli-Vergani G. De-novo autoimmune hepatitis after liver transplantation. *Lancet* 1998; **351**: 409-413 [PMID: 9482295 DOI: 10.1016/S0140-6736(97)06478-7]
- Gupta P, Hart J, Millis JM, Cronin D, Brady L. De novo hepatitis with autoimmune antibodies and atypical histology: a rare cause of late graft dysfunction after pediatric liver transplantation. *Transplantation* 2001; **71**: 664-668 [PMID: 11292299]
- Andries S, Casamayou L, Sempoux C, Bulet M, Reding R, Bernard Otte J, Buts JP, Sokal E. Posttransplant immune hepatitis in pediatric liver transplant recipients: incidence and maintenance therapy with azathioprine. *Transplantation* 2001; **72**: 267-272 [PMID: 11477351]
- Hernandez HM, Kovarik P, Whittington PF, Alonso EM. Autoimmune hepatitis as a late complication of liver transplantation. *J Pediatr Gastroenterol Nutr* 2001; **32**: 131-136 [PMID: 11321380]
- Petz W, Sonzogni A, Bertani A, Spada M, Lucianetti A, Colledan M, Gridelli B. A cause of late graft dysfunction after pediatric liver transplantation: de novo autoimmune hepatitis. *Transplant Proc* 2002; **34**: 1958-1959 [PMID: 12176643 DOI: 10.1016/S0041-1345(02)03137-8]
- Miyagawa-Hayashino A, Haga H, Sakurai T, Shirase T, Manabe T, Egawa H. De novo autoimmune hepatitis affecting allograft but not the native liver in auxiliary partial orthotopic liver transplantation. *Transplantation* 2003; **76**: 271-272 [PMID: 12865828 DOI: 10.1097/01.TP.0000072338.88465.59]
- Gibelli NE, Tannuri U, Mello ES, Cançado ER, Santos MM, Ayoub AA, Maksoud-Filho JG, Velhote MC, Silva MM, Pinho-Apezzato ML, Maksoud JG. Successful treatment of de novo autoimmune hepatitis and cirrhosis after pediatric liver transplantation. *Pediatr Transplant* 2006; **10**: 371-376 [PMID: 16677364 DOI: 10.1111/j.1399-3046.2005.00470.x]
- Evans HM, Kelly DA, McKiernan PJ, Hübscher S. Progressive histological damage in liver allografts following pediatric liver transplantation. *Hepatology* 2006; **43**: 1109-1117 [PMID: 16628633 DOI: 10.1002/hep.21152]
- Riva S, Sonzogni A, Bravi M, Bertani A, Alessio MG, Candusso M, Stroppa P, Melzi ML, Spada M, Gridelli B, Colledan M, Torre G. Late graft dysfunction and autoantibodies after liver transplantation in children: preliminary results of an Italian experience. *Liver Transpl* 2006; **12**: 573-577 [PMID: 16555335 DOI: 10.1002/lt.20673]
- Oya H, Sato Y, Yamamoto S, Kobayashi T, Watanabe T, Kokai H, Hatakeyama K. De novo autoimmune hepatitis after living donor liver transplantation in a 25-day-old newborn baby: a case report. *Transplant Proc* 2009; **41**: 433-434 [PMID: 19249573 DOI: 10.1016/j.transproceed.2008.10.031]
- Cho JM, Kim KM, Oh SH, Lee YJ, Rhee KW, Yu E. De novo autoimmune hepatitis in Korean children after liver transplantation: a single institution's experience. *Transplant Proc* 2011; **43**: 2394-2396 [PMID: 21839275 DOI: 10.1016/j.transproceed.2011.05.030]
- Pongpaibul A, Venick RS, McDiarmid SV, Lassman CR. Histopathology of de novo autoimmune hepatitis. *Liver Transpl* 2012; **18**: 811-818 [PMID: 22378542 DOI: 10.1002/lt.23422]
- Ekong UD, McKiernan P, Martinez M, Lobritto S, Kelly D, Ng VL, Alonso EM, Avitzur Y. Long-term outcomes of de novo autoimmune hepatitis in pediatric liver transplant recipients. *Pediatr Transplant* 2017; **21** [PMID: 28556542 DOI: 10.1111/ptr.12945]
- Jones DE, James OF, Portmann B, Burt AD, Williams R, Hudson M. Development of autoimmune hepatitis following liver transplantation for primary biliary cirrhosis. *Hepatology* 1999; **30**: 53-57 [PMID: 10385638 DOI: 10.1002/hep.510300103]
- Heneghan MA, Portmann BC, Norris SM, Williams R, Muiesan P, Rela M, Heaton ND, O'Grady JG. Graft dysfunction mimicking autoimmune hepatitis following liver transplantation in adults. *Hepatology* 2001; **34**: 464-470 [PMID: 11526530 DOI: 10.1053/jhep.2001.26756]
- Salcedo M, Vaquero J, Banares R, Rodríguez-Mahou M, Alvarez E, Vicario JL, Hernández-Albujar A, Tiscar JL, Rincón D, Alonso S, De Diego A, Clemente G. Response to steroids in de novo autoimmune hepatitis after liver transplantation. *Hepatology* 2002; **35**: 349-356 [PMID: 11826408 DOI: 10.1053/jhep.2002.31167]
- Aguilera I, Sousa JM, Gavilán F, Bernardos A, Wichmann I, Nuñez-Roldán A. Glutathione S-transferase T1 mismatch constitutes a risk factor for de novo immune hepatitis after liver transplantation. *Liver Transpl* 2004; **10**: 1166-1172 [PMID: 15350010 DOI: 10.1002/lt.20209]
- Miyagawa-Hayashino A, Haga H, Egawa H, Hayashino Y, Sakurai T, Minamiguchi S, Tanaka K, Manabe T. Outcome and risk factors of de novo autoimmune hepatitis in living-donor liver transplantation. *Transplantation* 2004; **78**: 128-135 [PMID: 15257051]
- Tsuji H, Hiramatsu K, Minato H, Kaneko S, Nakanuma Y. Auxiliary partial orthotopic liver transplantation with de novo autoimmune hepatitis in the allograft and leftover primary biliary cirrhosis in the native liver. *Semin Liver Dis* 2005; **25**: 371-377 [PMID: 16143952 DOI: 10.1055/s-2005-916328]
- Rodríguez-Díaz Y, Reyes-Rodríguez R, Dorta-Francisco MC,

- Aguilera I, Perera-Molinero A, Moneva-Arce E, Aviles-Ruiz JF. De novo autoimmune hepatitis following liver transplantation for primary biliary cirrhosis. *Transplant Proc* 2006; **38**: 1467-1470 [PMID: 16797335 DOI: 10.1016/j.transproceed.2006.03.071]
- 22 **Yoshizawa K**, Shirakawa H, Ichijo T, Umemura T, Tanaka E, Kiyosawa K, Imagawa E, Matsuda K, Hidaka E, Sano K, Nakazawa Y, Ikegami T, Hashikura Y, Miyagawa S, Ota M, Nakano M. De novo autoimmune hepatitis following living-donor liver transplantation for primary biliary cirrhosis. *Clin Transplant* 2008; **22**: 385-390 [PMID: 18190552 DOI: 10.1111/j.1399-0012.2007.00787.x]
- 23 **Fiel MI**, Agarwal K, Stanca C, Elhajj N, Kontorinis N, Thung SN, Schiano TD. Posttransplant plasma cell hepatitis (de novo autoimmune hepatitis) is a variant of rejection and may lead to a negative outcome in patients with hepatitis C virus. *Liver Transpl* 2008; **14**: 861-871 [PMID: 18508382 DOI: 10.1002/lt.21447]
- 24 **Zhang Y**, Wang B, Wang T. De novo autoimmune hepatitis with centrilobular necrosis following liver transplantation for primary biliary cirrhosis: a case report. *Transplant Proc* 2010; **42**: 3854-3857 [PMID: 21094869 DOI: 10.1016/j.transproceed.2010.08.062]
- 25 **Montano-Loza AJ**, Vargas-Vorackova F, Ma M, Bain VG, Burak K, Kumar T, Mason AL. Incidence and risk factors associated with de novo autoimmune hepatitis after liver transplantation. *Liver Int* 2012; **32**: 1426-1433 [PMID: 22712495 DOI: 10.1111/j.1478-3231.2012.02832.x]
- 26 **Banff Working Group**, Demetris AJ, Adeyi O, Bellamy CO, Clouston A, Charlotte F, Czaja A, Daskal I, El-Monayeri MS, Fontes P, Fung J, Gridelli B, Guido M, Haga H, Hart J, Honsova E, Hubscher S, Itoh T, Jhala N, Jungmann P, Khettry U, Lassman C, Ligato S, Lunz JG 3rd, Marcos A, Minervini MI, Mölne J, Nalesnik M, Nasser I, Neil D, Ochoa E, Pappo O, Randhawa P, Reinholt FP, Ruiz P, Sebagh M, Spada M, Sonzogni A, Tsamandas AC, Wernerson A, Wu T, Yilmaz F. Liver biopsy interpretation for causes of late liver allograft dysfunction. *Hepatology* 2006; **44**: 489-501 [PMID: 16871565 DOI: 10.1002/hep.21280]
- 27 **Shaikh OS**, Demetris AJ. Idiopathic posttransplantation hepatitis? *Liver Transpl* 2007; **13**: 943-946 [PMID: 17600346 DOI: 10.1002/lt.21202]
- 28 **Schreuder TC**, Hübscher SG, Neuberger J. Autoimmune liver diseases and recurrence after orthotopic liver transplantation: what have we learned so far? *Transpl Int* 2009; **22**: 144-152 [PMID: 18662365 DOI: 10.1111/j.1432-2277.2008.00729]
- 29 **Hübscher SG**. Antibody-mediated rejection in the liver allograft. *Curr Opin Organ Transplant* 2012; **17**: 280-286 [PMID: 22569512 DOI: 10.1097/MOT.0b013e328353584c]
- 30 **Hübscher SG**. What is the long-term outcome of the liver allograft? *J Hepatol* 2011; **55**: 702-717 [PMID: 21426919 DOI: 10.1016/j.jhep.2011.03.005]
- 31 **Sebagh M**, Castillo-Rama M, Azoulay D, Coilly A, Delvart V, Allard MA, Dos Santos A, Johanet C, Roque-Afonso AM, Saliba F, Duclos-Vallée JC, Samuel D, Demetris AJ. Histologic findings predictive of a diagnosis of de novo autoimmune hepatitis after liver transplantation in adults. *Transplantation* 2013; **96**: 670-678 [PMID: 23982338 DOI: 10.1097/TP.0b013e31829eda7f]
- 32 **Vukotic R**, Vitale G, D'Errico-Grigioni A, Muratori L, Andreone P. De novo autoimmune hepatitis in liver transplant: State-of-the-art review. *World J Gastroenterol* 2016; **22**: 2906-2914 [PMID: 26973387 DOI: 10.3748/wjg.v22.i10.2906]
- 33 **Nagai S**, Ito M, Kamei H, Nakamura T, Ando H, Kiuchi T. Indirect immunohistochemical evaluation of graft fibrosis and interface hepatitis after pediatric liver transplantation. *Pediatr Transplant* 2010; **14**: 342-350 [PMID: 19744282 DOI: 10.1111/j.1399-3046.2009.01234.x]
- 34 **Demetris AJ**, Sebagh M. Plasma cell hepatitis in liver allografts: Variant of rejection or autoimmune hepatitis? *Liver Transpl* 2008; **14**: 750-755 [PMID: 18508366 DOI: 10.1002/lt.21518]
- 35 **Solez K**, Colvin RB, Racusen LC, Haas M, Sis B, Mengel M, Halloran PF, Baldwin W, Banfi G, Collins AB, Cosio F, David DS, Drachenberg C, Einecke G, Fogo AB, Gibson IW, Grotz D, Iskandar SS, Kraus E, Lerut E, Mannon RB, Mihatsch M, Nankivell BJ, Nicleleit V, Papadimitriou JC, Randhawa P, Regele H, Renaudin K, Roberts I, Seron D, Smith RN, Valente M. Banff 07 classification of renal allograft pathology: updates and future directions. *Am J Transplant* 2008; **8**: 753-760 [PMID: 18294345 DOI: 10.1111/j.1600-6143.2008.02159.x]
- 36 **Aguilera I**, Sousa JM, Gavilan F, Gomez L, Alvarez-Márquez A, Núñez-Roldán A. Complement component 4d immunostaining in liver allografts of patients with de novo immune hepatitis. *Liver Transpl* 2011; **17**: 779-788 [PMID: 21425430 DOI: 10.1002/lt.22302]
- 37 **Trivedi A**, Schiano TD, Ward SC, Thung SN, Fiel MI, Levitsky J. C4d is present in portal venules in plasma cell hepatitis and may also be used as a predictor for its development. *Hepatology* 2014; **60**: 457A
- 38 **Deshpande V**, Zen Y, Chan JK, Yi EE, Sato Y, Yoshino T, Klöppel G, Heathcote JG, Khosroshahi A, Ferry JA, Aalberse RC, Bloch DB, Brugge WR, Bateman AC, Carruthers MN, Chari ST, Cheuk W, Cornell LD, Fernandez-Del Castillo C, Forcione DG, Hamilos DL, Kamisawa T, Kasashima S, Kawa S, Kawano M, Lauwers GY, Masaki Y, Nakanuma Y, Notohara K, Okazaki K, Ryu JK, Saeki T, Sahani DV, Smyrk TC, Stone JR, Takahira M, Webster GJ, Yamamoto M, Zamboni G, Umehara H, Stone JH. Consensus statement on the pathology of IgG4-related disease. *Mod Pathol* 2012; **25**: 1181-1192 [PMID: 22596100 DOI: 10.1038/modpathol.2012.72]
- 39 **Tamaro G**, Sonzogni A, Torre G. Monitoring "de novo" autoimmune hepatitis (LKM positive) by serum type-IV collagen after liver transplant: a paediatric case. *Clin Chim Acta* 2001; **310**: 25-29 [PMID: 11485751 DOI: 10.1016/S0009-8981(01)00509-5]
- 40 **Tripathi D**, Neuberger J. Autoimmune hepatitis and liver transplantation: indications, results, and management of recurrent disease. *Semin Liver Dis* 2009; **29**: 286-296 [PMID: 19676001 DOI: 10.1055/s-0029-1233531]
- 41 **Wichmann I**, Aguilera I, Sousa JM, Bernardos A, García Núñez EJ, Vigil E, Magariño R, Magariño I, Torres A, Núñez-Roldán A. Antibodies against glutathione S-transferase T1 in non-solid organ transplanted patients. *Transfusion* 2006; **46**: 1505-1509 [PMID: 16965576 DOI: 10.1111/j.1537-2995.2006.00938.x]
- 42 **Aguilera I**, Wichmann I, Sousa JM, Bernardos A, Franco E, García-Lozano JR, Núñez-Roldán A. Antibodies against glutathione S-transferase T1 (GSTT1) in patients with de novo immune hepatitis following liver transplantation. *Clin Exp Immunol* 2001; **126**: 535-539 [PMID: 11737073]
- 43 **Rodriguez-Mahou M**, Salcedo M, Fernandez-Cruz E, Tiscar JL, Bañares R, Clemente G, Vicario JL, Alvarez E, Rodriguez-Sainz C. Antibodies against glutathione S-transferase T1 (GSTT1) in patients with GSTT1 null genotype as prognostic marker: long-term follow-up after liver transplantation. *Transplantation* 2007; **83**: 1126-1129 [PMID: 17452905 DOI: 10.1097/01.tp.0000259963.47350.da]
- 44 **Czaja AJ**. Diagnosis, pathogenesis, and treatment of autoimmune hepatitis after liver transplantation. *Dig Dis Sci* 2012; **57**: 2248-2266 [PMID: 22562533 DOI: 10.1007/s10620-012-2179-3]
- 45 **Bellamy CO**. Complement C4d immunohistochemistry in the assessment of liver allograft biopsy samples: applications and pitfalls. *Liver Transpl* 2011; **17**: 747-750 [PMID: 21542127]
- 46 **Haga H**, Egawa H, Fujimoto Y, Ueda M, Miyagawa-Hayashino A, Sakurai T, Okuno T, Koyanagi I, Takada Y, Manabe T. Acute humoral rejection and C4d immunostaining in ABO blood type-incompatible liver transplantation. *Liver Transpl* 2006; **12**: 457-464 [PMID: 16498648 DOI: 10.1002/lt.20652]
- 47 **Kozlowski T**, Rubinas T, Nicleleit V, Woosley J, Schmitz J, Collins D, Hayashi P, Passannante A, Andreoni K. Liver allograft antibody-mediated rejection with demonstration of sinusoidal C4d staining and circulating donor-specific antibodies. *Liver Transpl* 2011; **17**: 357-368 [PMID: 21445918 DOI: 10.1002/lt.22233]
- 48 **Dumortier J**, Scoazec JY, Guillaud O, Hequet O, Hervieu V, Boillot O. Treatment of severe refractory de novo auto-immune hepatitis after liver transplantation with plasmapheresis. *Clin Res Hepatol Gastroenterol* 2015; **39**: e83-e85 [PMID: 26070570 DOI: 10.1016/j.clinre.2015.04.003]

- 49 **Wozniak LJ**, Hickey MJ, Venick RS, Vargas JH, Farmer DG, Busuttil RW, McDiarmid SV, Reed EF. Donor-specific HLA Antibodies Are Associated With Late Allograft Dysfunction After Pediatric Liver Transplantation. *Transplantation* 2015; **99**: 1416-1422 [PMID: 26038872 DOI: 10.1097/TP.0000000000000796]
- 50 **Ward SC**, Schiano TD, Thung SN, Fiel MI. Plasma cell hepatitis in hepatitis C virus patients post-liver transplantation: case-control study showing poor outcome and predictive features in the liver explant. *Liver Transpl* 2009; **15**: 1826-1833 [PMID: 19938116 DOI: 10.1002/lt.21949]
- 51 **Neuberger J**. An update on liver transplantation: A critical review. *J Autoimmun* 2016; **66**: 51-59 [PMID: 26350881 DOI: 10.1016/j.jaut.2015.08.021]
- 52 **Mack CL**. What Causes Biliary Atresia? Unique Aspects of the Neonatal Immune System Provide Clues to Disease Pathogenesis. *Cell Mol Gastroenterol Hepatol* 2015; **1**: 267-274 [PMID: 26090510 DOI: 10.1016/j.jcmgh.2015.04.001]
- 53 **Alvarez F**. Is biliary atresia an immune mediated disease? *J Hepatol* 2013; **59**: 648-650 [PMID: 23792027 DOI: 10.1016/j.jhep.2013.06.006]
- 54 **Pang SY**, Dai YM, Zhang RZ, Chen YH, Peng XF, Fu J, Chen ZR, Liu YF, Yang LY, Wen Z, Yu JK, Liu HY. Autoimmune liver disease-related autoantibodies in patients with biliary atresia. *World J Gastroenterol* 2018; **24**: 387-396 [PMID: 29391761 DOI: 10.3748/wjg.v24.i3.387]
- 55 **Muraji T**, Ohtani H, Ieiri S. Unique manifestations of biliary atresia provide new immunological insight into its etiopathogenesis. *Pediatr Surg Int* 2017; **33**: 1249-1253 [PMID: 29022092 DOI: 10.1007/s00383-017-4155-7]
- 56 **Martínez-Bravo MJ**, Sánchez B, Sousa JM, Acevedo MJ, Gómez-Bravo MA, Núñez-Roldán A, Aguilera I. T-cell allorecognition of donor glutathione S-transferase T1 in plasma cell-rich rejection. *World J Hepatol* 2017; **9**: 1115-1124 [PMID: 29026463 DOI: 10.4254/wjh.v9.i27.1115]
- 57 **Vergani D**, Mieli-Vergani G. The impact of autoimmunity on hepatocytes. *Semin Liver Dis* 2007; **27**: 140-151 [PMID: 17520514 DOI: 10.1055/s-2007-979467]
- 58 **Aguado-Domínguez E**, Gómez L, Sousa JM, Gómez-Bravo MA, Núñez-Roldán A, Aguilera I. Identification of the cellular components involved in de novo immune hepatitis: a quantitative immunohistochemical analysis. *J Transl Med* 2018; **16**: 62 [PMID: 29534755 DOI: 10.1186/s12967-018-1440-8]
- 59 **Eguchi S**, Takatsuki M, Hidaka M, Tajima Y, Zen Y, Nakanuma Y, Kanematsu T. De novo autoimmune hepatitis after living donor liver transplantation is unlikely to be related to immunoglobulin subtype 4-related immune disease. *J Gastroenterol Hepatol* 2008; **23**: e165-e169 [PMID: 18505414 DOI: 10.1111/j.1440-1746.2008.05347.x]
- 60 **Castillo-Rama M**, Sebah M, Sasatomi E, Randhawa P, Isse K, Salgarkar AD, Ruppert K, Humar A, Demetris AJ. "Plasma cell hepatitis" in liver allografts: identification and characterization of an IgG4-rich cohort. *Am J Transplant* 2013; **13**: 2966-2977 [PMID: 24011021 DOI: 10.1111/ajt.12413]
- 61 **Aguilera I**, Martínez-Bravo MJ, Sousa JM, Pozo-Borrego AJ, Núñez-Roldán A. IgG subclass profile among anti-Glutathione S-transferase T1 antibodies in post-transplant de novo immune hepatitis. *Clin Transplant* 2016; **30**: 210-217 [PMID: 26663521 DOI: 10.1111/ctr.12675]
- 62 **Ekong UD**, Mathew J, Melin-Aldana H, Wang D, Alonso EM. Successful resolution of inflammation and increased regulatory T cells in sirolimus-treated post-transplant allograft hepatitis. *Pediatr Transplant* 2012; **16**: 165-175 [PMID: 22360400 DOI: 10.1111/j.1399-3046.2012.01648.x]
- 63 **Edmunds C**, Ekong UD. Autoimmune Liver Disease Post-Liver Transplantation: A Summary and Proposed Areas for Future Research. *Transplantation* 2016; **100**: 515-524 [PMID: 26447505 DOI: 10.1097/TP.0000000000000922]
- 64 **Martínez-Bravo MJ**, Calderón-Cabrera C, Márquez-Malaver FJ, Rodríguez N, Guijarro M, Espigado I, Núñez-Roldán A, Pérez-Simón JA, Aguilera I. Mismatch on glutathione S-transferase T1 increases the risk of graft-versus-host disease and mortality after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant* 2014; **20**: 1356-1362 [PMID: 24844856 DOI: 10.1016/j.bbmt.2014.05.008]
- 65 **Aguado-Domínguez E**, Sousa JM, Pérez-Simón JA, Aguilera I. Clinical association of anti-glutathione S-transferase T1 antibodies and de novo immune hepatitis after hematopoietic cell transplantation. *Dig Liver Dis* 2018; **50**: 418-419 [PMID: 29409781 DOI: 10.1016/j.dld.2017.12.033]
- 66 **Kerkar N**, Yanni G. 'De novo' and 'recurrent' autoimmune hepatitis after liver transplantation: A comprehensive review. *J Autoimmun* 2016; **66**: 17-24 [PMID: 26377632 DOI: 10.1016/j.jaut.2015.08.017]
- 67 **Arterbery AS**, Osafo-Addo A, Avitzur Y, Ciarleglio M, Deng Y, Lobritto SJ, Martínez M, Hafler DA, Kleinewietfeld M, Ekong UD. Production of Proinflammatory Cytokines by Monocytes in Liver-Transplanted Recipients with De Novo Autoimmune Hepatitis Is Enhanced and Induces TH1-like Regulatory T Cells. *J Immunol* 2016; **196**: 4040-4051 [PMID: 27183637 DOI: 10.4049/jimmunol.150227]
- 68 **Aguilera I**, Sousa JM, Praena JM, Gómez-Bravo MA, Núñez-Roldán A. Choice of calcineurin inhibitor may influence the development of de novo immune hepatitis associated with anti-GSTT1 antibodies after liver transplantation. *Clin Transplant* 2011; **25**: 207-212 [PMID: 20236132 DOI: 10.1111/j.1399-0012.2010.01221.x]

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Endoscopic diagnosis of sessile serrated adenoma/polyp with and without dysplasia/carcinoma

Takashi Murakami, Naoto Sakamoto, Akihito Nagahara

Takashi Murakami, Naoto Sakamoto, Akihito Nagahara, Department of Gastroenterology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

ORCID number: Takashi Murakami (0000-0001-7419-589X); Naoto Sakamoto (0000-0002-2143-0734); Akihito Nagahara (0000-0001-5979-4384).

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Correspondence to: Takashi Murakami, MD, PhD, Assistant Professor, Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. t-murakm@juntendo.ac.jp
Telephone: +81-3-38133111
Fax: +81-3-38138862

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Abstract

Sessile serrated adenoma/polyps (SSA/Ps) are early precursor lesions in the serrated neoplasia pathway, which results in colorectal carcinomas with *BRAF* mutations, methylation for DNA repair genes, a CpG island methylator phenotype, and high levels of microsatellite instability. Some of these lesions can rapidly become dysplastic or invasive carcinomas that exhibit high lymphatic invasion and lymph node metastasis potentials. Detecting serrated lesions, including SSA/Ps with and without dysplasia/carcinoma, is critical, but SSA/Ps can be difficult to detect, are inconsistently identified by endoscopists and pathologists, and are often incompletely resected. Therefore, SSA/Ps are considered to be major contributors to "interval cancers". If colonoscopists can identify the specific endoscopic characteristics of SSA/Ps, their detection and the effectiveness of colonoscopy may improve. Here, the endoscopic features of SSA/Ps with and without dysplasia/carcinoma, including the characteristics determined using magnifying endoscopy, are reviewed in the context of previous reports. Endoscopically, these subtle polyps are like hyperplastic polyps, because they are slightly elevated and pale. Unlike hyperplastic polyps, SSA/Ps are usually larger than 5 mm, frequently covered by a thin layer called the "mucus cap", and are more commonly located in the proximal colon. Magnifying narrow-band imaging findings, which include dark spots inside the crypts and varicose microvascular vessels, in addition to the type II-open pit patterns detected using magnifying chromoendoscopy, effectively differentiate SSA/Ps from hyperplastic polyps. The lesions' endoscopic characteristics, which include their (semi)pedunculated morphologies, double elevations, central depressions, and reddishness, and the use of magnifying endoscopy, might help to detect dysplasia/carcinoma within SSA/Ps. Greater awareness may promote further research into improving the detection, identification, and complete

resection rates of SSA/Ps with and without dysplasia/carcinoma and reduce the interval cancer rates.

Key words: Sessile serrated adenoma/polyp; Invasive carcinoma arising from sessile serrated adenoma/polyp; Serrated neoplasia pathway; Endoscopic diagnosis; Sessile serrated adenoma/polyp with cytological dysplasia

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Core tip: The endoscopic features of sessile serrated adenoma/polyps (SSA/Ps) with and without dysplasia/carcinoma are reviewed. Conventional endoscopic characteristics, including a proximal location, a slightly elevated morphology, a pale color, and a mucus cap, are useful for diagnosing SSA/Ps. Magnifying narrow-band imaging, which detects dark spots inside the crypts and varicose microvascular vessels, and magnifying chromoendoscopy, which identifies the type II-open pit pattern, are also effective for differentiating between SSA/Ps and hyperplastic polyps. Furthermore, the lesions' endoscopic characteristics, which include their (semi)pedunculated morphologies, double elevations, central depressions, and reddishness, and the use of magnifying endoscopy, might help to detect dysplasia/carcinoma within SSA/Ps.

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INTRODUCTION

Colorectal serrated lesions were called "hyperplastic polyps", and they were not considered to be malignant^[1,2]. Torlakovic *et al*^[3] described abnormal proliferations in colorectal serrated polyps that resembled hyperplastic polyps superficially, but could be distinguished histologically based on their abnormal architectural features, and they introduced the term "sessile serrated adenoma". Currently, these polyps are categorized as sessile serrated adenoma/polyp (SSA/P) in accordance with the World Health Organization's recommendations^[4]. The typical histology of an SSA/P in a representative case is shown in Figure 1.

SSA/Ps are early precursor lesions in the serrated neoplasia pathway, which results in colorectal carcinomas with high levels of microsatellite instability^[5-7]. Recent studies have shown associations between SSA/Ps with and without dysplasia or carcinoma and the methylation or loss of protein expression for DNA repair genes, including *MLH1*^[3,6,8-12], a CpG island methylator phenotype^[5,6,8,10], *BRAF* mutations^[5,6,8-17], and a lack of genetic alterations in *CTNNB1*, which is the gene that

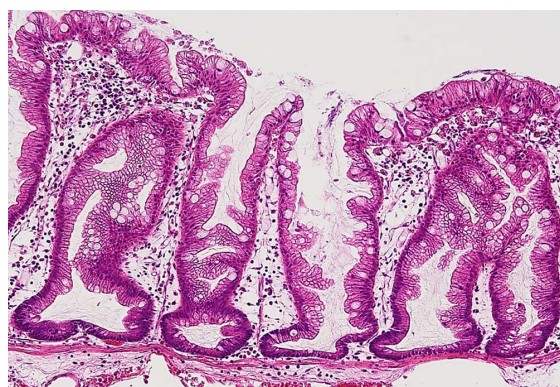


Figure 1 Typical histology of a sessile serrated adenoma/polyp. Crypts with a serrated architecture include those that are irregularly dilated, branch irregularly, and are horizontally arranged (basal).

codes for β -catenin protein^[17]. This pathway is thought to be distinct from the conventional adenoma-carcinoma pathway in which adenomas progress to invasive colorectal carcinomas as a result of a series of genetic alterations, including adenomatous polyposis coli (*APC*) and *KRAS* mutations^[6,8,13,14,18,19].

Some researchers^[20-22] have suggested that some serrated lesions might progress rapidly to dysplasia or invasive carcinomas. Furthermore, we reported that the submucosal invasive carcinomas that arose in SSA/Ps exhibited higher potentials for lymphatic invasion and lymph node metastasis than their conventional counterparts that arose from tubular adenomas^[23]. Therefore, the detection of serrated lesions, including SSA/Ps with and without dysplasia, is critical. However, SSA/Ps can be difficult to detect, are inconsistently identified by endoscopists and pathologists, and are often incompletely resected^[24-27]. Therefore, SSA/Ps are major contributors to the failure of colonoscopy to prevent proximal colonic cancer^[28-30], and they account for 5%-7% of the colorectal cancers that occur in the interval between a complete colonoscopy and surveillance, that is, "interval cancer"^[31-33]. The identification of the specific endoscopic characteristics of SSA/Ps by colonoscopists may improve their detection and, eventually, may enhance the effectiveness of colonoscopy. Some studies have investigated the endoscopic features of SSA/Ps without dysplasia^[34-38], and we clarified the endoscopic characteristics of SSA/Ps that had advanced histology^[39].

Here, the endoscopic features of SSA/Ps with and without dysplasia or carcinoma are reviewed in the context of previous reports, including the features detected using magnifying endoscopy.

DIAGNOSIS OF SSA/P USING CONVENTIONAL WHITE-LIGHT ENDOSCOPY

Generally, hyperplastic polyps are traditionally considered non-neoplastic, but SSA/Ps have malignant potential to progress to invasive carcinomas. Therefore,

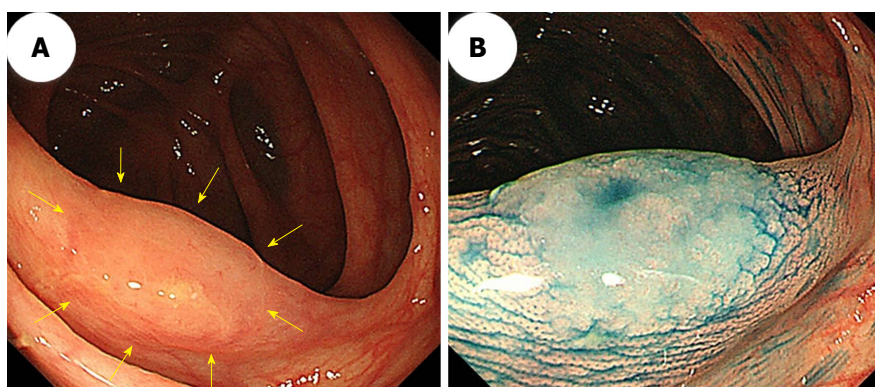


Figure 2 A sessile serrated adenoma/polyp in the transverse colon that measured 13 mm. A: An image from conventional colonoscopy showing the lesion's location (arrows); B: An image from chromoendoscopy following indigo carmine dye spraying.

differentiating an SSA/P from a hyperplastic polyp is clinically important to determine the necessity of an endoscopic resection or to provide support for a recommendation of a surveillance interval^[40,41]. Typical hyperplastic polyps are highly prevalent, diminutive sessile polyps that are most commonly located in the sigmoid colon and rectum, and identifying them endoscopically is not particularly difficult^[42]. SSA/Ps are subtle polyps, and their endoscopic findings are similar to those associated with hyperplastic polyps, which include a slightly elevated morphology and a pale color. However, in contrast to hyperplastic polyps, SSA/Ps are usually larger than 5 mm, frequently covered by a thin layer called a "mucus cap"^[4,34,43,44], and are more commonly located in the proximal colon^[14,45]. Conversely, although SSA/Ps are difficult to detect because of their slightly elevated morphology, adhesion of mucus in the proximal colon can be one of the most useful clues for SSA/P detection. Additionally, using white-light endoscopy, Hazewinkel *et al.*^[37] described the presence of indistinct borders and a cloud-like surface, and showed that these were independently predictive endoscopic characteristics that were associated with the histology of SSA/Ps. Figure 2 shows representative endoscopic images of SSA/Ps.

ENDOSCOPIC DIAGNOSIS OF SSA/P USING NARROW-BAND IMAGING

Difficulties distinguishing between an SSA/P and a hyperplastic polyp are commonly encountered. Many authors have used image-enhanced endoscopy to characterize polyps^[46], which involves the use of innovative optical technologies, such as narrow-band imaging (NBI)^[47-50]. Bile appears as a bright red fluid using NBI. When a tenacious mucus cap covers SSA/Ps, the mucus cap is clearly viewed using NBI. Therefore, NBI enhances the visibility of SSA/Ps that have mucus caps, which are usually an intense red color^[34] (Figure 3A and B).

Furthermore, NBI often reveals small dark dots inside the openings to the crypts of SSA/Ps^[37]; these are thought to indicate crypt dilations, which are a key

histological feature of SSA/Ps. The presence of these dark spots inside the crypts might help endoscopists to differentiate between premalignant SSA/Ps and hyperplastic polyps during colonoscopy^[37,38] (Figure 3C and D). Hazewinkel *et al.*^[37] have reported that in white-light endoscopy, indistinct borders and cloud-like surface are two independent predictive characteristics of SSA/P, while in NBI, it is possible to discern an irregular shape and dark spots inside the crypts. The sensitivities, specificities, and overall accuracies determined using white-light endoscopy were 75%, 79%, and 77%, respectively, and those determined using NBI were 89%, 96%, and 93%, respectively^[37].

Magnifying NBI can enhance the visibility of the microvessels on a lesion's surface. Yamada *et al.*^[51] conducted a multivariate analysis, demonstrated that dilated and branching vessels, defined as thickened capillary vessels with branching that is observed on the surface, had a 2.3-fold odds ratio among SSA/Ps compared with hyperplastic polyps. They stated that when dilated and branching vessels, a proximal location, and a tumor size of ≥ 10 mm were combined, the positive predictive value exceeded 90%. Additionally, Uraoka *et al.*^[52] reported that the presence of varicose microvascular vessels, which were found using magnifying NBI, was useful for differentiating between SSA/Ps and hyperplastic polyps. Unlike the blood vessels around the glands of the superficial mucosal layer such as dilated and branching vessels, varicose microvascular vessels are characterized by the observation of blood vessels running throughout the deep mucosal layer. The presence of varicose microvascular vessels had a significantly higher specificity (88%) for predicting a diagnosis of SSA/P (Figure 3E and F).

DIAGNOSIS OF SSA/P USING MAGNIFYING CHROMOENDOSCOPY

Magnifying chromoendoscopy, which uses indigo carmine or crystal violet staining, follows careful conventional endoscopic examinations. Kudo *et al.*^[53,54] proposed a classification of colorectal lesions' pit patterns that is associated with the lesions' histologic characteristics.

Table 1 Distinct endoscopic characteristics between sessile serrated adenoma/polyps and hyperplastic polyps

	SSA/Ps	Hyperplastic polyps
Conventional endoscopic features		
Location	Proximal	Distal
Size of tumor	> 5 mm	≤ 5 mm
Color	Pale	Pale
Morphology	Flat elevated	Flat elevated
Mucus cap	Yes	No
Endoscopic features by using NBI	Irregular shape Small dark dots Dilated and branching vessels Varicose microvascular vessels	-
Magnifying chromoendoscopic features	Type II-open pit pattern	Type II pit pattern

SSA/P: Sessile serrated adenoma/polyp; NBI: Narrow-band imaging.

As previously explained^[54-56], magnifying colonoscopy is useful for differentiating between neoplastic and nonneoplastic lesions, and for assessing early colorectal cancers' depths of invasion. Both hyperplastic polyps and SSA/Ps have type II pit patterns. Recently, the type II-open pit pattern has been described as a hallmark of SSA/Ps (sensitivity: 66%; specificity: 97%)^[35]. Like the small dark dots detected using NBI, a type II-open pit pattern detected using magnifying chromoendoscopy is thought to indicate crypt dilation, which is one of the major histological features of SSA/Ps (Figure 4).

Distinct endoscopic characteristics between SSA/Ps and hyperplastic polyps are summarized in Table 1.

ENDOSCOPIC DETECTION OF SSA/P

The detection of SSA/Ps requires careful colonoscopy. As stated above, because most SSA/Ps are slightly flat-elevated and have subtle mucosal features, SSA/Ps are difficult to detect with endoscopy, and could easily be missed. Therefore, bowel preparation must be excellent. Potential SSA/Ps are initially considered at long view and investigated at close-up view. At long view, the presence of SSA/P is suspected when there is a patch that appears nodular, reddish, covered with mucus, and/or circled by fine debris. Then such a lesion must be approached and the mucosa washed. Finally, at close-up view, using white light and under NBI, the surface pattern and vessels are examined.

Recently, some studies^[57,58] have shown that image-enhanced endoscopy such as NBI might increase the detection of serrated lesions in the proximal colon, although the results did not reach significance. Therefore, image-enhanced endoscopy currently cannot be recommended as a detection tool for SSA/P. Additional studies assessing SSA/P detection rates with image-enhanced endoscopy are needed.

ENDOSCOPIC DIAGNOSIS OF SSA/P WITH DYSPLASIA/CARCINOMA

SSA/Ps with advanced histology, including cytologic dysplasia or minimally invasive carcinomas, are rare.

Indeed, a previous study's findings showed that the frequencies of cytologic dysplasia and invasive carcinomas among SSA/P lesions were 14% and 1.0%, respectively^[59]. The findings from another study showed that three (0.7%) high-grade dysplasias and one (0.2%) submucosal invasive carcinoma were detected among 430 SSA/Ps^[60]. Therefore, only a few studies have investigated the endoscopic characteristics of SSA/Ps with dysplasia or carcinoma in detail^[39,61,62]. We demonstrated that SSA/Ps without dysplasia (354 of 414; 86%) and SSA/Ps with dysplasia or carcinomas (40 of 48; 83%) were frequently located in the proximal colon^[39]. Furthermore, we showed a stepwise increase in the median size of the SSA/Ps that accompanied their dysplastic progression, specifically, from a 10-mm SSA/P that did not have dysplasia to a 12-mm SSA/P with cytologic dysplasia and a 19-mm SSA/P with an invasive carcinoma, but 19 of 48 (39.6%) SSA/Ps with dysplasia or carcinomas measured ≤ 10 mm^[39]. The findings from a study by Goldstein^[20] showed that the median size of eight SSA/Ps with focal invasive adenocarcinomas or high-grade dysplasia was 8.5 mm (range: 6-12 mm). Another study's findings^[63] showed that among eight SSA/Ps with intramucosal carcinomas, submucosal carcinomas, or advanced carcinomas, the largest diameter was ≤ 10 mm. Therefore, SSA/P with dysplasia/carcinoma must be attended to even if the lesion measures 10 mm or less.

Macroscopically, a mucus cap was found in almost all of the SSA/P lesions, including the SSA/Ps with and without dysplasia or carcinoma, in our study^[39], suggesting that a mucus cap may be one of the strongest markers of an SSA/P. Additionally, (semi)pedunculated morphologies, double elevations, central depressions, and reddishness were found more frequently in SSA/Ps with dysplasia (17.1%, 63.4%, 9.8%, and 39.0%, respectively) or carcinoma (28.6%, 57.1%, 28.6%, and 85.7%, respectively) than the frequencies at which these features were found in SSA/Ps without dysplasia (4.6%, 4.6%, 3.9%, and 3.4%, respectively). The presence of at least one of these four markers had a high sensitivity (91.7%) for the identification of dysplasia or a carcinoma within an SSA/P; the specificity was

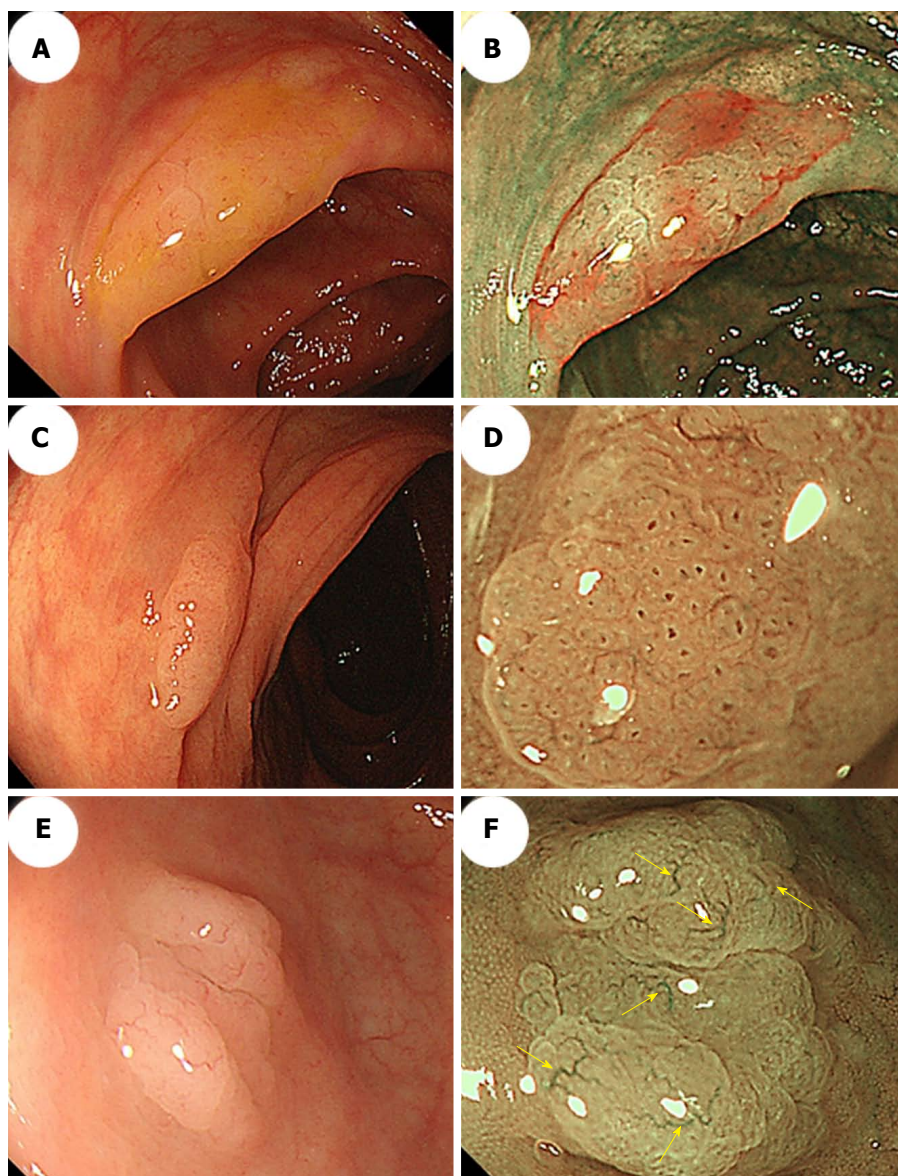


Figure 3 Morphologic characteristics of sessile serrated adenoma/polyps. A: Conventional endoscopy revealed a flat-elevated lesion with a 20-mm diameter that was covered with a mucus cap in the transverse colon. B: Narrow-band imaging (NBI) showed that the SSA/P in (A) was covered with a mucus cap that appeared intensely red. C: Conventional endoscopy showed a flat-elevated lesion with a 14-mm diameter in the ascending colon. D: Magnifying NBI of the SSA/P in (C) revealed dark spots inside the crypts in part of the lesion. E: A conventional endoscopic image shows a flat-elevated pale colored lesion with a 10-mm diameter in the cecum. F: Magnifying NBI of the SSA/P in (E) revealed varicose microvascular vessels (arrows) in part of the lesion. SSA/P: Sessile serrated adenoma/polyp.

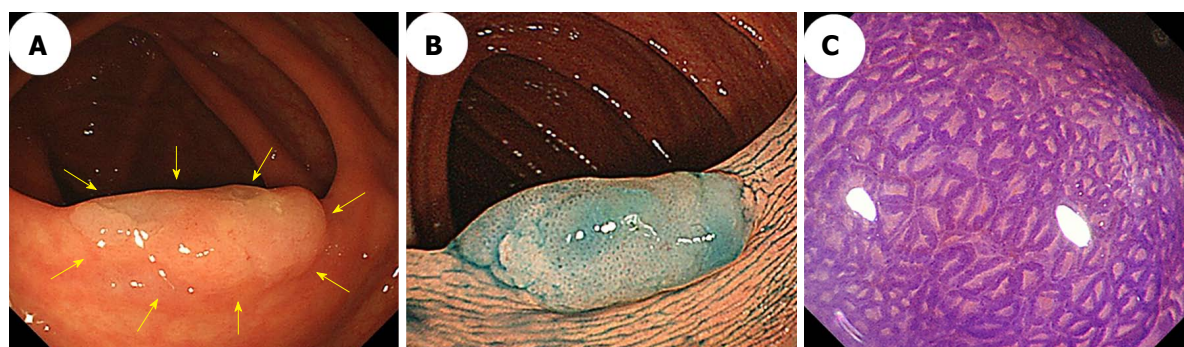


Figure 4 Conventional colonoscopic image (A) and a chromoendoscopic image (B) following indigo carmine dye spraying show an 18-mm sessile serrated adenoma/polyp with a mucus cap that was in the transverse colon (arrows). C: Magnifying chromoendoscopy using crystal violet staining identified a type II-open pit pattern in the lesion.

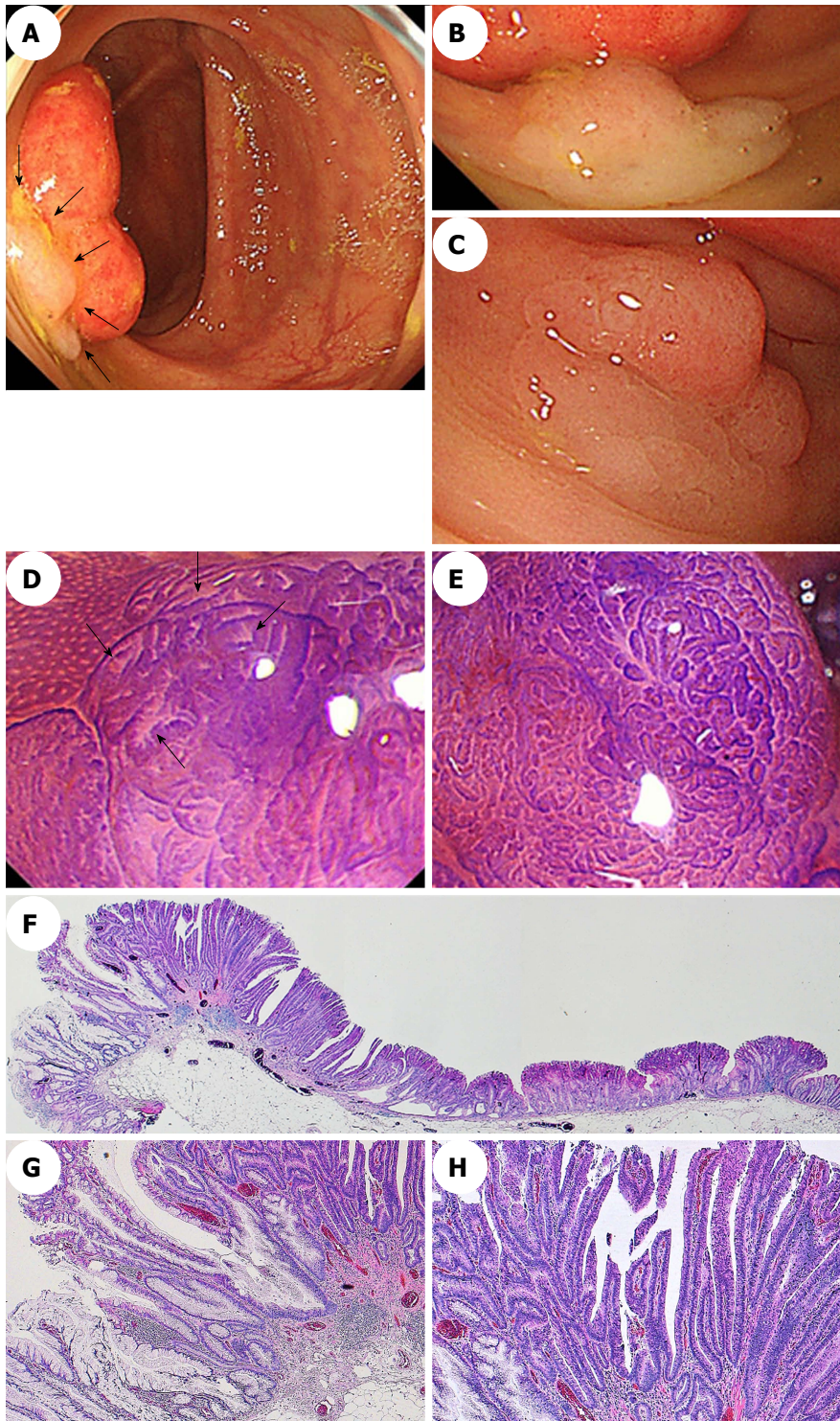


Figure 5 Endoscopic images of a sessile serrated adenoma/polyp with high-grade cytologic dysplasia in a representative case. A-C: A conventional endoscopic view using white-light imaging. A: An endoscopic image shows a pale-color, flat-elevated lesion covered with mucus at the ascending colon (arrows). B: The lesion is covered with mucus cap. C: After washing the target lesion to sufficiently remove mucus, a flat-elevated lesion that had a 13-mm diameter and a dome-shaped double elevation can be clearly seen. The dome-shaped area is slightly red-colored. D and E: Magnifying chromoendoscopic views using crystal violet staining. D: A type II-open pit pattern is partly evident in the edge of the lesion (arrows). E: Type VI-mild pit pattern consisting of areas with irregular pits can be observed at the dome-shaped area. We endoscopically diagnosed the lesion as an SSA/P with cytologic dysplasia, and achieved an en bloc resection by performing an endoscopic mucosal resection. F-H: Histopathologic findings with hematoxylin-eosin staining of the resected specimen. G: Crypts with a serrated architecture exhibit irregularly dilated crypts, irregularly branching crypts, and horizontally arranged basal crypts, corresponding to SSA/P. H: A high-power view shows conventional adenomatous high-grade dysplasia with cytological atypia and architectural dysplasia in the dome-shaped area. The lesion was pathologically consistent with an SSA/P with high-grade cytologic dysplasia. SSA/P: Sessile serrated adenoma/polyp.

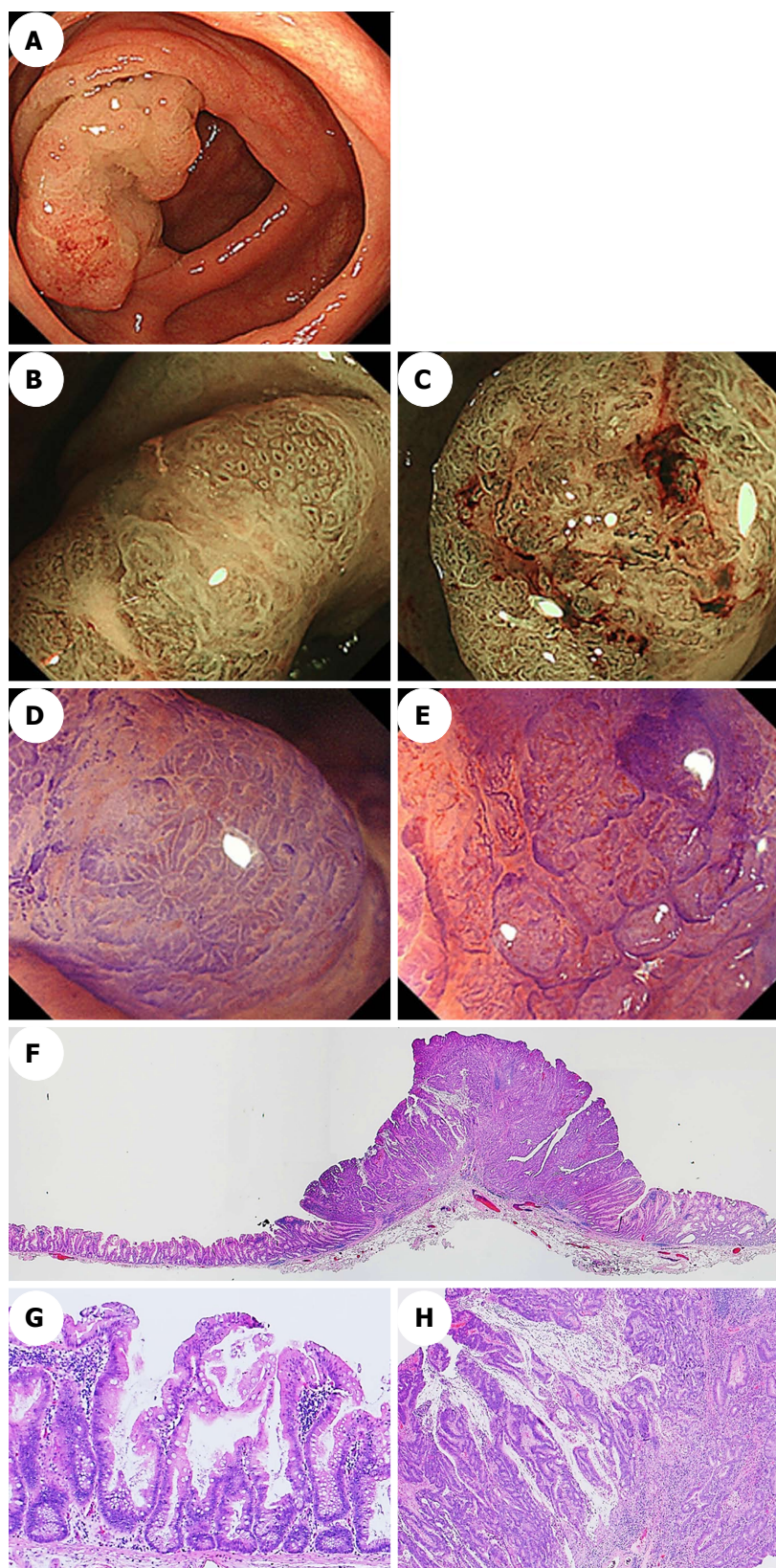


Figure 6 Endoscopic images of a sessile serrated adenoma/polyp with an invasive carcinoma in a representative case. A: A conventional endoscopic image captured using white-light imaging shows a red 55-mm semipedunculated lesion in the ascending colon. B and C: Magnifying narrow-band imaging revealed dark spots inside the crypts on an edge of the lesion and irregular vessel patterns over a large part of the lesion, respectively. D and E: Magnifying chromoendoscopy using crystal violet staining; D: A high-powered view of the marginal zone, the dilated openings of the crypts have a type II-open pit pattern; E: A high-powered view of the middle region in which a type VI-severe pit pattern is evident. We endoscopically diagnosed the lesion as a carcinoma associated with an SSA/P, and achieved an en bloc resection by performing an endoscopic submucosal dissection. F-H: Histopathologic findings with hematoxylin-eosin staining of the resected specimen; G: Crypts with a serrated architecture exhibiting irregularly dilated crypts and irregularly branching crypts, corresponding to SSA/P; H: Well to moderately differentiated adenocarcinomas invade the submucosa with extracellular mucin production. The lesion was pathologically consistent with an invasive submucosal adenocarcinoma associated with an SSA/P. SSA/P: Sessile serrated adenoma/polyp.

85.3%. These findings suggested that the endoscopic characteristics, including a (semi)pedunculated morphology, a double elevation, a central depression, and reddishness, may be useful for accurately diagnosing the presence of advanced histology within an SSA/P.

Magnifying chromoendoscopy is also useful for detecting components associated with dysplasia or a carcinoma within an SSA/P. We found that a type II-open pit pattern was present in SSA/Ps without dysplasia and in SSA/Ps with dysplasia or carcinomas, which indicates that a type II-open pit pattern may be strongly suggestive of the presence of SSA/P components^[39]. Furthermore, the type II pit pattern only was detected in all of the cases who had SSA/Ps without dysplasia, whereas type II and other pit patterns, including mixtures of III_L, IV, VI, or V_N, were found in most of the SSA/Ps with dysplasia or carcinoma. Moreover, all of the cases who had SSA/Ps with invasive carcinomas had the VI or V_N pit patterns (invasive patterns), which were consistent with the depths of invasion. Accordingly, determining the pit patterns using magnifying endoscopy can effectively assess the depth of invasion of early colorectal cancers that arise from SSA/Ps. Figures 5 and 6 show representative endoscopic images of SSA/Ps with dysplasia or carcinoma.

Finally, there is one important point that must be kept in mind when observing SSA/Ps using colonoscopy. Most SSA/Ps were covered with rich mucus, and subtle endoscopic findings were difficult to detect when sticky mucus was present. After washing the target lesion to sufficiently remove mucus, endoscopic findings such as (semi)pedunculated morphology, double elevation, central depression, and reddishness should be assessed, and pit pattern analysis must be performed.

CONCLUSION

Conventional endoscopic characteristics, including a proximal location, a slightly elevated morphology, a pale color, and a mucus cap, are useful for diagnosing SSA/Ps. Magnifying endoscopy with NBI, which detects dark spots inside the crypts and varicose microvascular vessels, and magnifying chromoendoscopy, which identifies the type II-open pit pattern, are also effective for differentiating between SSA/Ps and hyperplastic polyps. Furthermore, a lesion's endoscopic characteristics, for example, a (semi)pedunculated morphology, a double elevation, a central depression, and reddishness, in addition to the use of magnifying endoscopy, might be useful for identifying dysplasia or a carcinoma within an SSA/P. Greater awareness may promote further research into improving the detection, recognition, and complete resection rates of SSA/Ps with and without dysplasia or carcinoma and reduce the interval cancer rates.

REFERENCES

- 1 Lane N. The precursor tissue of ordinary large bowel cancer. *Cancer Res* 1976; **36**: 2669-2672 [PMID: 1277173]

- 2 Jørgensen H, Mogensen AM, Svendsen LB. Hyperplastic polyposis of the large bowel. Three cases and a review of the literature. *Scand J Gastroenterol* 1996; **31**: 825-830 [PMID: 8858755]
- 3 Torlakovic E, Skovlund E, Snover DC, Torlakovic G, Nesland JM. Morphologic reappraisal of serrated colorectal polyps. *Am J Surg Pathol* 2003; **27**: 65-81 [PMID: 12502929]
- 4 Snover DC, Ahnen DJ, Burt RW. Serrated polyps of the colon and rectum and serrated polyposis. In: Bosman FT, Carneiro F, Hruban RH, Theise ND, eds. WHO classification of tumours of the digestive system. Lyon: IARC Press; 2010: 160-165
- 5 Kambara T, Simms LA, Whitehall VL, Spring KJ, Wynter CV, Walsh MD, Barker MA, Arnold S, McGivern A, Matsubara N, Tanaka N, Higuchi T, Young J, Jass JR, Leggett BA. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. *Gut* 2004; **53**: 1137-1144 [PMID: 15247181 DOI: 10.1136/gut.2003.037671]
- 6 O'Brien MJ, Yang S, Mack C, Xu H, Huang CS, Mulcahy E, Amoroso M, Farraye FA. Comparison of microsatellite instability, CpG island methylation phenotype, BRAF and KRAS status in serrated polyps and traditional adenomas indicates separate pathways to distinct colorectal carcinoma end points. *Am J Surg Pathol* 2006; **30**: 1491-1501 [PMID: 17122504 DOI: 10.1097/01.pas.0000213313.36306.85]
- 7 Patil DT, Shadrach BL, Rybicki LA, Leach BH, Pai RK. Proximal colon cancers and the serrated pathway: a systematic analysis of precursor histology and BRAF mutation status. *Mod Pathol* 2012; **25**: 1423-1431 [PMID: 22684223 DOI: 10.1038/modpathol.2012.98]
- 8 Kim YH, Kakar S, Cun L, Deng G, Kim YS. Distinct CpG island methylation profiles and BRAF mutation status in serrated and adenomatous colorectal polyps. *Int J Cancer* 2008; **123**: 2587-2593 [PMID: 18798261 DOI: 10.1002/ijc.23840]
- 9 Sandmeier D, Benhattar J, Martin P, Bouzourene H. Serrated polyps of the large intestine: a molecular study comparing sessile serrated adenomas and hyperplastic polyps. *Histopathology* 2009; **55**: 206-213 [PMID: 19694828 DOI: 10.1111/j.1365-2559.2009.03356.x]
- 10 Kim KM, Lee EJ, Ha S, Kang SY, Jang KT, Park CK, Kim JY, Kim YH, Chang DK, Odze RD. Molecular features of colorectal hyperplastic polyps and sessile serrated adenoma/polyps from Korea. *Am J Surg Pathol* 2011; **35**: 1274-1286 [PMID: 21836485 DOI: 10.1097/PAS.0b013e318224cd2e]
- 11 Dhir M, Yachida S, Van Neste L, Glöckner SC, Jeschke J, Pappou EP, Montgomery EA, Herman JG, Baylin SB, Iacobuzio-Donahue C, Ahuja N. Sessile serrated adenomas and classical adenomas: an epigenetic perspective on premalignant neoplastic lesions of the gastrointestinal tract. *Int J Cancer* 2011; **129**: 1889-1898 [PMID: 21154739 DOI: 10.1002/ijc.25847]
- 12 Murakami T, Mitomi H, Saito T, Takahashi M, Sakamoto N, Fukui N, Yao T, Watanabe S. Distinct WNT/β-catenin signaling activation in the serrated neoplasia pathway and the adenoma-carcinoma sequence of the colorectum. *Mod Pathol* 2015; **28**: 146-158 [PMID: 24925057 DOI: 10.1038/modpathol.2014.41]
- 13 Jass JR, Baker K, Zlobec I, Higuchi T, Barker M, Buchanan D, Young J. Advanced colorectal polyps with the molecular and morphological features of serrated polyps and adenomas: concept of a 'fusion' pathway to colorectal cancer. *Histopathology* 2006; **49**: 121-131 [PMID: 16879389 DOI: 10.1111/j.1365-2559.2006.02466.x]
- 14 Spring KJ, Zhao ZZ, Karamatic R, Walsh MD, Whitehall VL, Pike T, Simms LA, Young J, James M, Montgomery GW, Appleyard M, Hewett D, Togashi K, Jass JR, Leggett BA. High prevalence of sessile serrated adenomas with BRAF mutations: a prospective study of patients undergoing colonoscopy. *Gastroenterology* 2006; **131**: 1400-1407 [PMID: 17101316 DOI: 10.1053/j.gastro.2006.08.038]
- 15 Carr NJ, Mahajan H, Tan KL, Hawkins NJ, Ward RL. Serrated and non-serrated polyps of the colorectum: their prevalence in an unselected case series and correlation of BRAF mutation analysis with the diagnosis of sessile serrated adenoma. *J Clin Pathol* 2009; **62**: 516-518 [PMID: 19126563 DOI: 10.1136/jcp.2008.061960]
- 16 Fujita K, Yamamoto H, Matsumoto T, Hirahashi M, Gushima

- M, Kishimoto J, Nishiyama K, Taguchi T, Yao T, Oda Y. Sessile serrated adenoma with early neoplastic progression: a clinicopathologic and molecular study. *Am J Surg Pathol* 2011; **35**: 295-304 [PMID: 21263251 DOI: 10.1097/PAS.0b013e318205df36]
- 17 **Yachida S**, Mudali S, Martin SA, Montgomery EA, Iacobuzio-Donahue CA. Beta-catenin nuclear labeling is a common feature of sessile serrated adenomas and correlates with early neoplastic progression after BRAF activation. *Am J Surg Pathol* 2009; **33**: 1823-1832 [PMID: 19745699 DOI: 10.1097/PAS.0b013e3181b6da19]
 - 18 **Powell SM**, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW. APC mutations occur early during colorectal tumorigenesis. *Nature* 1992; **359**: 235-237 [PMID: 1528264 DOI: 10.1038/359235a0]
 - 19 **Miyoshi Y**, Nagase H, Ando H, Horii A, Ichii S, Nakatsuru S, Aoki T, Miki Y, Mori T, Nakamura Y. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. *Hum Mol Genet* 1992; **1**: 229-233 [PMID: 1338904]
 - 20 **Goldstein NS**. Small colonic microsatellite unstable adenocarcinomas and high-grade epithelial dysplasias in sessile serrated adenoma polypectomy specimens: a study of eight cases. *Am J Clin Pathol* 2006; **125**: 132-145 [PMID: 16483002]
 - 21 **Snover DC**. Update on the serrated pathway to colorectal carcinoma. *Hum Pathol* 2011; **42**: 1-10 [PMID: 20869746 DOI: 10.1016/j.humpath.2010.06.002].
 - 22 **Bettington M**, Walker N, Clouston A, Brown I, Leggett B, Whitehall V. The serrated pathway to colorectal carcinoma: current concepts and challenges. *Histopathology* 2013; **62**: 367-386 [PMID: 23339363 DOI: 10.1111/his.12055]
 - 23 **Murakami T**, Mitomi H, Yao T, Saito T, Shibuya T, Sakamoto N, Osada T, Watanabe S. Distinct histopathological characteristics in colorectal submucosal invasive carcinoma arising in sessile serrated adenoma/polyp and conventional tubular adenoma. *Virchows Arch* 2018; **472**: 383-393 [PMID: 28929387 DOI: 10.1007/s00428-017-2234-8]
 - 24 **Hetzel JT**, Huang CS, Coukos JA, Omstead K, Cerda SR, Yang S, O'Brien MJ, Farrar FA. Variation in the detection of serrated polyps in an average risk colorectal cancer screening cohort. *Am J Gastroenterol* 2010; **105**: 2656-2664 [PMID: 20717107 DOI: 10.1038/ajg.2010.315]
 - 25 **Kahi CJ**, Hewett DG, Norton DL, Eckert GJ, Rex DK. Prevalence and variable detection of proximal colon serrated polyps during screening colonoscopy. *Clin Gastroenterol Hepatol* 2011; **9**: 42-46 [PMID: 20888435 DOI: 10.1016/j.cgh.2010.09.013]
 - 26 **Pohl H**, Srivastava A, Bensen SP, Anderson P, Rothstein RI, Gordon SR, Levy LC, Toor A, Mackenzie TA, Rosch T, Robertson DJ. Incomplete polyp resection during colonoscopy-results of the complete adenoma resection (CARE) study. *Gastroenterology* 2013; **144**: 74-80.e1 [PMID: 23022496 DOI: 10.1053/j.gastro.2012.09.043]
 - 27 **de Wijkerslooth TR**, Stoop EM, Bossuyt PM, Tytgat KM, Dees J, Mathus-Vliegen EM, Kuipers EJ, Fockens P, van Leerdam ME, Dekker E. Differences in proximal serrated polyp detection among endoscopists are associated with variability in withdrawal time. *Gastrointest Endosc* 2013; **77**: 617-623 [PMID: 23321338 DOI: 10.1016/j.gie.2012.10.018]
 - 28 **Baxter NN**, Goldwasser MA, Paszat LF, Saskin R, Urbach DR, Rabeneck L. Association of colonoscopy and death from colorectal cancer. *Ann Intern Med* 2009; **150**: 1-8 [PMID: 19075198]
 - 29 **Brenner H**, Hoffmeister M, Arndt V, Stegmaier C, Altenhofen L, Haug U. Protection from right- and left-sided colorectal neoplasms after colonoscopy: population-based study. *J Natl Cancer Inst* 2010; **102**: 89-95 [PMID: 20042716 DOI: 10.1093/jnci/djp436]
 - 30 **Singh H**, Nugent Z, Demers AA, Kliever EV, Mahmud SM, Bernstein CN. The reduction in colorectal cancer mortality after colonoscopy varies by site of the cancer. *Gastroenterology* 2010; **139**: 1128-1137 [PMID: 20600026 DOI: 10.1053/j.gastro.2010.06.052]
 - 31 **Sawhney MS**, Farrar WD, Gudiseva S, Nelson DB, Lederle FA, Rector TS, Bond JH. Microsatellite instability in interval colon cancers. *Gastroenterology* 2006; **131**: 1700-1705 [PMID: 17087932 DOI: 10.1053/j.gastro.2006.10.022]
 - 32 **Araim MA**, Sawhney M, Sheikh S, Anway R, Thyagarajan B, Bond JH, Shaikat A. CIMP status of interval colon cancers: another piece to the puzzle. *Am J Gastroenterol* 2010; **105**: 1189-1195 [PMID: 20010923 DOI: 10.1038/ajg.2009.699]
 - 33 **Cooper GS**, Xu F, Barnholtz Sloan JS, Schlachter MD, Koroukian SM. Prevalence and predictors of interval colorectal cancers in medicare beneficiaries. *Cancer* 2012; **118**: 3044-3052 [PMID: 21989586 DOI: 10.1002/cncr.26602]
 - 34 **Tadepalli US**, Feihel D, Miller KM, Itzkowitz SH, Freedman JS, Kornacki S, Cohen LB, Bamji ND, Bodian CA, Aisenberg J. A morphologic analysis of sessile serrated polyps observed during routine colonoscopy (with video). *Gastrointest Endosc* 2011; **74**: 1360-1368 [PMID: 22018553 DOI: 10.1016/j.gie.2011.08.008]
 - 35 **Kimura T**, Yamamoto E, Yamano HO, Suzuki H, Kamimae S, Nojima M, Sawada T, Ashida M, Yoshikawa K, Takagi R, Kato R, Harada T, Suzuki R, Maruyama R, Kai M, Imai K, Shinomura Y, Sugai T, Toyota M. A novel pit pattern identifies the precursor of colorectal cancer derived from sessile serrated adenoma. *Am J Gastroenterol* 2012; **107**: 460-469 [PMID: 22233696 DOI: 10.1038/ajg.2011.457]
 - 36 **Ishigooka S**, Nomoto M, Obinata N, Oishi Y, Sato Y, Nakatsu S, Suzuki M, Ikeda Y, Maehata T, Kimura T, Watanabe Y, Nakajima T, Yamano HO, Yasuda H, Itoh F. Evaluation of magnifying colonoscopy in the diagnosis of serrated polyps. *World J Gastroenterol* 2012; **18**: 4308-4316 [PMID: 22969193 DOI: 10.3748/wjg.v18.i32.4308]
 - 37 **Hazewinkel Y**, López-Cerón M, East JE, Rastogi A, Pellisé M, Nakajima T, van Eeden S, Tytgat KM, Fockens P, Dekker E. Endoscopic features of sessile serrated adenomas: validation by international experts using high-resolution white-light endoscopy and narrow-band imaging. *Gastrointest Endosc* 2013; **77**: 916-924 [PMID: 23433877 DOI: 10.1016/j.gie.2012.12.018]
 - 38 **Yamashina T**, Takeuchi Y, Uedo N, Aoi K, Matsuura N, Nagai K, Matsui F, Ito T, Fujii M, Yamamoto S, Hanaoka N, Higashino K, Ishihara R, Tomita Y, Iishi H. Diagnostic features of sessile serrated adenoma/polyps on magnifying narrow band imaging: a prospective study of diagnostic accuracy. *J Gastroenterol Hepatol* 2015; **30**: 117-123 [PMID: 25088839 DOI: 10.1111/jgh.12688]
 - 39 **Murakami T**, Sakamoto N, Ritsuno H, Shibuya T, Osada T, Mitomi H, Yao T, Watanabe S. Distinct endoscopic characteristics of sessile serrated adenoma/polyp with and without dysplasia/carcinoma. *Gastrointest Endosc* 2017; **85**: 590-600 [PMID: 27663716 DOI: 10.1016/j.gie.2016.09.018]
 - 40 **Rex DK**, Ahnen DJ, Baron JA, Batts KP, Burke CA, Burt RW, Goldblum JR, Guillem JG, Kahi CJ, Kalady MF, O'Brien MJ, Odze RD, Ogino S, Parry S, Snover DC, Torlakovic EE, Wise PE, Young J, Church J. Serrated lesions of the colorectum: review and recommendations from an expert panel. *Am J Gastroenterol* 2012; **107**: 1315-1329; quiz 1314, 1330 [PMID: 22710576 DOI: 10.1038/ajg.2012.161]
 - 41 **Lieberman DA**, Rex DK, Winawer SJ, Giardiello FM, Johnson DA, Levin TR. Guidelines for colonoscopy surveillance after screening and polypectomy: a consensus update by the US Multi-Society Task Force on Colorectal Cancer. *Gastroenterology* 2012; **143**: 844-857 [PMID: 22763141 DOI: 10.1053/j.gastro.2012.06.001]
 - 42 **Morson BC**. Precancerous lesions of the colon and rectum. Classification and controversial issues. *JAMA* 1962; **179**: 316-321 [PMID: 14476361]
 - 43 **Boparai KS**, van den Broek FJ, van Eeden S, Fockens P, Dekker E. Hyperplastic polyposis syndrome: a pilot study for the differentiation of polyps by using high-resolution endoscopy, autofluorescence imaging, and narrow-band imaging. *Gastrointest Endosc* 2009; **70**: 947-955 [PMID: 19595313 DOI: 10.1016/j.gie.2009.03.1172]
 - 44 **Gurudu SR**, Heigh RI, De Petris G, Heigh EG, Leighton JA, Pasha SF, Malagon IB, Das A. Sessile serrated adenomas: demographic, endoscopic and pathological characteristics. *World J Gastroenterol* 2010; **16**: 3402-3405 [PMID: 20632442]
 - 45 **Higuchi T**, Sugihara K, Jass JR. Demographic and pathological characteristics of serrated polyps of colorectum. *Histopathology* 2005;

- 47: 32-40 [PMID: 15982321 DOI: 10.1111/j.1365-2559.2005.02180.x]
- 46 **Tajiri H**, Niwa H. Proposal for a consensus terminology in endoscopy: how should different endoscopic imaging techniques be grouped and defined? *Endoscopy* 2008; **40**: 775-778 [PMID: 18698532 DOI: 10.1055/s-2008-1077507]
- 47 **Sano Y**, Horimatsu T, Fu KI, Katagiri A, Muto M, Ishikawa H. Magnifying observation of microvascular architecture of colorectal lesions using a narrow band imaging system. *Dig Endosc* 2006; **18**: S44-S51 [DOI: 10.1111/j.0915-5635.2006.00621.x]
- 48 **Chiu HM**, Chang CY, Chen CC, Lee YC, Wu MS, Lin JT, Shun CT, Wang HP. A prospective comparative study of narrow-band imaging, chromoendoscopy, and conventional colonoscopy in the diagnosis of colorectal neoplasia. *Gut* 2007; **56**: 373-379 [PMID: 17005766 DOI: 10.1136/gut.2006.099614]
- 49 **Uraoka T**, Sano Y, Saito Y, Saito H, Matsuda T, Yamamoto K. Narrow-band imaging for improving colorectal adenoma detection: appropriate system function settings are required. *Gut* 2009; **58**: 604-605 [PMID: 19299388 DOI: 10.1136/gut.2008.157164]
- 50 **Nakao Y**, Saito S, Ohya T, Aihara H, Arihiro S, Kato T, Ikegami M, Tajiri H. Endoscopic features of colorectal serrated lesions using image-enhanced endoscopy with pathological analysis. *Eur J Gastroenterol Hepatol* 2013; **25**: 981-988 [PMID: 23820237 DOI: 10.1097/MEG.0b013e3283614b2b]
- 51 **Yamada M**, Sakamoto T, Otake Y, Nakajima T, Kuchiba A, Taniguchi H, Sekine S, Kushima R, Rambaran H, Parra-Blanco A, Fujii T, Matsuda T, Saito Y. Investigating endoscopic features of sessile serrated adenomas/polyps by using narrow-band imaging with optical magnification. *Gastrointest Endosc* 2015; **82**: 108-117 [PMID: 25840928 DOI: 10.1016/j.gie.2014.12.037]
- 52 **Uraoka T**, Higashi R, Horii J, Harada K, Hori K, Okada H, Mizuno M, Tomoda J, Ohara N, Tanaka T, Chiu HM, Yahagi N, Yamamoto K. Prospective evaluation of endoscopic criteria characteristic of sessile serrated adenomas/polyps. *J Gastroenterol* 2015; **50**: 555-563 [PMID: 25270966 DOI: 10.1007/s00535-014-0999-y]
- 53 **Kudo S**, Hirota S, Nakajima T, Hosobe S, Kusaka H, Kobayashi T, Himori M, Yagyu A. Colorectal tumours and pit pattern. *J Clin Pathol* 1994; **47**: 880-885 [PMID: 7962600]
- 54 **Kudo S**, Tamura S, Nakajima T, Yamano H, Kusaka H, Watanabe H. Diagnosis of colorectal tumorous lesions by magnifying endoscopy. *Gastrointest Endosc* 1996; **44**: 8-14 [PMID: 8836710]
- 55 **Kudo S**, Kashida H, Tamura T, Kogure E, Imai Y, Yamano H, Hart AR. Colonoscopic diagnosis and management of nonpolypoid early colorectal cancer. *World J Surg* 2000; **24**: 1081-1090 [PMID: 11036286]
- 56 **Kudo S**, Rubio CA, Teixeira CR, Kashida H, Kogure E. Pit pattern in colorectal neoplasia: endoscopic magnifying view. *Endoscopy* 2001; **33**: 367-373 [PMID: 11315901 DOI: 10.1055/s-2004-826104]
- 57 **Parikh ND**, Chaptini L, Njei B, Laine L. Diagnosis of sessile serrated adenomas/polyps with image-enhanced endoscopy: a systematic review and meta-analysis. *Endoscopy* 2016; **48**: 731-739 [PMID: 27223636 DOI: 10.1055/s-0042-107592]
- 58 **Rex DK**, Clodfelter R, Rahmani F, Fatima H, James-Stevenson TN, Tang JC, Kim HN, McHenry L, Kahi CJ, Rogers NA, Helper DJ, Sagi SV, Kessler WR, Wo JM, Fischer M, Kwo PY. Narrow-band imaging versus white light for the detection of proximal colon serrated lesions: a randomized, controlled trial. *Gastrointest Endosc* 2016; **83**: 166-171 [PMID: 25952085 DOI: 10.1016/j.gie.2015.03.1915]
- 59 **Lash RH**, Genta RM, Schuler CM. Sessile serrated adenomas: prevalence of dysplasia and carcinoma in 2139 patients. *J Clin Pathol* 2010; **63**: 681-686 [PMID: 20547691 DOI: 10.1136/jcp.2010.075507]
- 60 **Chino A**, Yamamoto N, Kato Y, Morishige K, Ishikawa H, Kishihara T, Fujisaki J, Ishikawa Y, Tamegai Y, Igarashi M. The frequency of early colorectal cancer derived from sessile serrated adenoma/polyps among 1858 serrated polyps from a single institution. *Int J Colorectal Dis* 2016; **31**: 343-349 [PMID: 26510850 DOI: 10.1007/s00384-015-2416-2]
- 61 **Bouwens MW**, van Herwaarden YJ, Winkens B, Rondagh EJ, de Ridder R, Riedl RG, Driessen A, Dekker E, Masclee AA, Sanduleanu S. Endoscopic characterization of sessile serrated adenomas/polyps with and without dysplasia. *Endoscopy* 2014; **46**: 225-235 [PMID: 24573732 DOI: 10.1055/s-0034-1364936]
- 62 **Burgess NG**, Pellise M, Nanda KS, Hourigan LF, Zanati SA, Brown GJ, Singh R, Williams SJ, Raftopoulos SC, Ormonde D, Moss A, Byth K, P'Ng H, McLeod D, Bourke MJ. Clinical and endoscopic predictors of cytological dysplasia or cancer in a prospective multicentre study of large sessile serrated adenomas/polyps. *Gut* 2016; **65**: 437-446 [PMID: 25731869 DOI: 10.1136/gutjnl-2014-308603]
- 63 **Ban S**, Mitomi H, Horiguchi H, Sato H, Shimizu M. Adenocarcinoma arising in small sessile serrated adenoma/polyp (SSA/P) of the colon: clinicopathological study of eight lesions. *Pathol Int* 2014; **64**: 123-132 [PMID: 24698422 DOI: 10.1111/pin.12147]

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Basic Study

Downregulation of Hes1 expression in experimental biliary atresia and its effects on bile duct structure

Rui-Zhong Zhang, Xin-Hao Zeng, Ze-Feng Lin, Ming-Fu, Yan-Lu Tong, Vincent CH Lui, Paul KH Tam, Jonathan R Lamb, Hui-Min Xia, Yan Chen

Rui-Zhong Zhang, Xin-Hao Zeng, Ze-Feng Lin, Ming-Fu, Yan-Lu Tong, Hui-Min Xia, Yan Chen, Department of Pediatric Surgery, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou 510623, Guangdong Province, China

Vincent CH Lui, Paul KH Tam, Yan Chen, Department of Surgery and Pathology, University of Hong Kong, Hong Kong, China

Jonathan R Lamb, Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, London SW7 2AZ, United Kingdom

ORCID number: Rui-Zhong Zhang (0000-0002-4954-7192); Xin-Hao Zeng (0000-0002-3879-9810); Ze-Feng Lin (0000-0001-7532-9175); Ming-Fu (0000-0003-1400-0258); Yan-Lu Tong (0000-0003-0650-1562); Vincent CH Lui (0000-0002-1758-8854); Paul KH Tam (0000-0003-1596-4120); Jonathan R Lamb (0000-0002-3879-9810); Hui-Min Xia (0000-0002-0103-1672); Yan Chen (0000-0002-2354-230X).

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Correspondence to: Yan Chen, PhD, Honorary Research Fellow, Senior Scientist, Department of Surgery and Pathology, University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong, China. yhcnc@hku.hk
Telephone: +86-852-28199602
Fax: +86-852-28199621

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Abstract

AIM

To analyze the expression and function of the notch

signaling target gene Hes1 in a rhesus rotavirus-induced mouse biliary atresia model.

METHODS

The morphologies of biliary epithelial cells in biliary atresia patients and in a mouse model were examined by immunohistochemical staining. Then, the differential expression of Notch signaling pathway-related molecules was investigated. Further, the effects of the siRNA-mediated inhibition of Hes1 expression were examined using a biliary epithelial cell 3D culture system.

RESULTS

Both immature (EpCAM⁺) and mature (CK19⁺) biliary epithelial cells were detected in the livers of biliary atresia patients without a ductile structure and in the mouse model with a distorted bile duct structure. The hepatic expression of transcripts for most Notch signaling molecules were significantly reduced on day 7 but recovered to normal levels by day 14, except for the target molecule Hes1, which still exhibited lower mRNA and protein levels. Expression of the Hes1 transcriptional co-regulator, RBP-J κ was also reduced. A 3D gel culture system promoted the maturation of immature biliary epithelial cells, with increased expression of CK19⁺ cells and the formation of a duct-like structure. The administration of Hes1 siRNA blocked this process. As a result, the cells remained in an immature state, and no duct-like structure was observed.

CONCLUSION

Our data indicated that Hes1 might contribute to the maturation and the cellular structure organization of biliary epithelial cells, which provides new insight into understanding the pathology of biliary atresia.

Key words: Biliary atresia; Rhesus rotavirus; Hes1; EpCAM; Epithelial cells 3D culture

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Core tip: Similar immature biliary epithelial cells and distorted bile ductules were observed in biliary atresia patients and a mouse biliary atresia model. Investigation of the Notch signaling pathway target gene Hes1 showed that mRNA and protein expression was reduced in mouse liver, which was partially due to reduced expression of transcriptional co-regulator RBP-J κ . In biliary epithelial cells 3D gel culture system, the administration of Hes1 siRNA blocked the maturation and duct-like structure formation process, which resulted in the cells still being in an immature state and no duct-like structure was observed.

Zhang RZ, Zeng XH, Lin ZF, Fu M, Tong YL, Lui VC, Tam PK, Lamb JR, Xia HM, Chen Y. Downregulation of Hes1 expression in experimental biliary atresia and its effects on bile duct structure. *World J Gastroenterol* 2018; 24(29): 3260-3272 Available from:

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INTRODUCTION

Biliary atresia (BA) is the most common cause of end-stage liver disease in infants, and it has a poor prognosis with a high fatality rate. It is characterized by persistent jaundice and progressive cholestasis that develops within weeks of birth. These symptoms are caused by progressive extrahepatic and/or intrahepatic bile duct inflammation and tissue fibrosis in both the bile ducts and the liver. Although the symptoms can be partially ameliorated by Kasai surgery and extensive post-operative care, some of the patients still develop cirrhosis and liver failure due to the progressive destruction of intrahepatic bile ducts and liver fibrosis^[1]. The incidence of BA in the USA is approximately 1:15000 live births, and the incidence is even higher in Asia at approximately 1:9600 live births^[2,3]. Without liver transplants, the long-term survival rate of BA is between 13% and 50%^[4]. Furthermore, the etiology of the disease is not fully understood. The birth defects and inflammation associated with viral infection and subsequent anti-viral immunity, which targets biliary epithelial cells (BEC), cause apoptosis and injury to the bile ducts. This results in a failure to reestablish the biliary system and progresses as tissue fibrosis, eventually leading to BA^[5].

The Notch signaling pathway plays a key role in the development and differentiation of hepatic stem cells into BECs in order to establish the biliary system^[6,7]. It is composed of a family of molecules, including Notch ligands, Notch receptors, DNA binding protein RBP-J κ and effectors, such as HES, HEY, and HERP, which mediate the biological function of Notch signaling. Mutations in the Notch ligand Jagged1 (Jag1) or receptor Notch2 in humans results in Alagille syndrome (AGS), which is characterized by biliary dysplasia and cholestasis^[8,9]. Similar findings have been observed in mice expressing null alleles of Jag1 or Notch2^[10], which suggests a mechanism of functional conservation. Hes1 is a target gene of the Notch signaling pathway and is essential for the tubular formation in the biliary epithelium^[11] and maintenance of the characteristics of the biliary epithelium^[12]. However, the effect of altered Hes1 expression in BA has not been established.

Pathological observations have suggested that the blockade of the extrahepatic bile duct is the major change in both patients with BA and the animal model of BA, and that damage to the intrahepatic bile duct further enhances bile accumulation in the liver, which promotes liver fibrosis. The number of intrahepatic biliary epithelial cells (IBECs) might differ from patient to patient, but immature cell status and malformed ductular structures were commonly observed in patient liver samples^[13]. Epithelial cell adhesion molecule (EpCAM) is expressed

in the embryonic stage of liver development^[14], and some studies have used it as an immature epithelial cell marker for cell isolation^[15]. CK19⁺ represents a biliary epithelial cell lineage marker. It is only weakly expressed in hepatocytes, whereas it is highly expressed in mature BECs^[16,17]. Using these two markers in this study, the presence of BECs was examined in rhesus rotavirus (RRV)-inoculated neonatal BA mice. The expression levels of components of the Notch signaling pathway, notably Hes1, were detected, and the effects of Hes1 loss of function on BEC morphological changes were evaluated in 3D cell culture.

MATERIALS AND METHODS

Reagents and antibodies

The rhesus rotavirus strain MMU 18006 (RRV) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, United States). The virus was amplified in MA104 cells, and the virus quantification was measured by a plaque assay method, as described previously^[18]. The antibodies used for immunohistochemical analyses of mouse tissue sections were rat anti-cytokeratin 19 (CK19, clone TROMA III) and rat anti-EpCAM (clone G8.8), both of which were purchased from DSHB (Developmental Studies Hybridoma Bank, Iowa City, IA, United States). Rabbit anti-human Hes1 and rabbit anti-human RBP-J κ were obtained from Cell Signaling Technology (Cell Signaling Technology Danvers, MA, United States). For human tissue section staining, rabbit anti-human CK19 and rabbit anti-human EpCAM were both obtained from Santa Cruz (Santa Cruz Biotechnology, Inc. Dallas, TX, United States). The RNA extraction kit and the reverse transcription reagents were purchased from Invitrogen (Life Technologies Limited, NT, Hong Kong), and Super Real Pre-Mix SYBR green was purchased from Tiangen [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China].

Human samples and pathological analysis

Clinical liver tissue samples from BA patients ($n = 20$) and disease controls [with choledochal cyst (CC), $n = 3$] were obtained from the Pathology Department of Guangzhou Women and Children's Medical Center. The data were analyzed anonymously. The diagnosis was established by both pathologists and physicians based on clinical observation during operations and a laboratory-based pathological analysis. Tissue fibrosis was demonstrated by Masson tricolor staining and Sirius Red staining (data not shown). The ages of the patients included in the study ranged from two to five months old. The experimental protocols were approved by the Medical Ethics Committee of Guangzhou Women and Children's Medical Center (2015090109).

Electron microscopy analysis

The tissues were fixed in neutral glutaraldehyde and osmic acid. After dehydration, the tissues were embedded in

resin. Tissue blocks were first sectioned at 1-2 microns with glass knives using an EM UC7 ultramicrotome (Leica, Buffalo Grove, IL, United States), and a reference was used to trim the blocks for thin sectioning. The appropriate blocks were then thinly sectioned at 50-70 nm. After drying on filter paper, the sections were stained with uranyl acetate and lead citrate for contrast. After drying, the grids were then viewed on a JEM-1400 microscope (JEOL USA, Inc., Peabody, MA, United States).

Infection of neonatal mice with rhesus rotavirus

Day 12.5 pregnant Balb/c mice aged between 10 and 12 wk were purchased from Guangdong Animal Experimental Centre, maintained under specific pathogen-free conditions and housed in a room with a 12-h dark-light cycle. All of the animal protocols were approved by The Institutional Animal Care and Use Committee of Guangzhou Medical University (2016-007).

To establish an experimental model of BA, neonatal mice were intraperitoneally injected within 24 h of birth with 20 μ L of either 4×10^4 PFU/mL RRV or MA104 cell culture supernatant as a control. Infected mice that died within the first two days or that were not fed by their mothers were excluded from further analysis. All appropriate measures were taken to minimize the pain and discomfort of the mice. The mice were weighed and examined daily, and, in general, the development of icterus of the skin not covered with fur and acholic stools were generally observed on days five to six after the injection, indicating a successful induction of BA. The mice were sacrificed by an overdose of pentobarbitone (200 mg/kg, i.p.). To obtain tissue samples, the animals were dissected under a microscope (Nikon SMZ1000), and the gross appearances of the livers and bile ducts were recorded. The tissues were harvested and stored at -80°C for RNA/protein isolation and in 10% formalin for histological sample preparation.

Histological and immunohistochemical analysis

Mouse and human liver samples were fixed and paraffin-embedded for immunostaining. Then, 4 μ m thick tissue sections were rehydrated and then stained with hematoxylin and eosin (HE) for histological analysis. For immuno-histochemistry, antigen retrieval was performed in citrate buffer [10 mmol/L, 0.01% Tween20, pH 6.0 for EpCAM or Tris-EDTA buffer (10 mmol/L Tris base, 1 mmol/L EDTA solution, pH 9.0 for CK19)], and for the removal of endogenous peroxidase, the samples were treated with 3% hydrogen peroxide. The sections were then incubated overnight at 4°C in blocking solution containing primary antibodies. Blocking solution without primary antibodies only was used as a control. After undergoing appropriate secondary antibody incubation, the immunostaining was completed using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, United States) and liquid 3,3'-diaminobenzidine (Dako). The results were analyzed using a Nikon microscope, and images were captured with NIS-Elements F4.0.

Table 1 Primers used in the experiments

Genes	5'→3'	Sequences
Mouse Jag1	Forward	TTCTCACTCAGGCATGATAAAACC
	Reverse	CATCTCTGGGACGACAGAACT
Mouse Dll1	Forward	CCCATCCGATTCCTTCG
	Reverse	GGTTTCTGTTGCGAGGTCATC
Mouse Notch1	Forward	CCCTTGCTCTGCCTAACGC
	Reverse	GGAGTCTCGGCATCGTTGG
Mouse Notch2	Forward	CTGTGAGCGGAATATCGACGA
	Reverse	ATAGCCTCCGTTTCGGTTGG
Mouse Hes1	Forward	CCAGCCAGTGTC AACACGA
	Reverse	AATGCCGGGAGCTATCTTCT
Mouse β -actin	Forward	AAACTGGAACGGTGAAGGTG
	Reverse	AGTGGGGTGGCTTTTAGGAT
Human Hes1	Forward	TCAACACGACACCGGATAAAC
	Reverse	GCCGCGAGCTATCTTCTCA
Human β -actin	Forward	TTAGTTCGTTACACCCCTTCTTGACA
	Reverse	CTGCACCTTCACCGTTCAGTTT

Gene expression profiling using quantitative PCR

The expression levels of Notch signaling-related molecules in the livers (harvested at 7 d and 14 d) of RRV-inoculated and saline control mice were determined using qPCR. The total RNA was extracted from a portion of each liver using TRIzol (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions, and the extracts were treated with DNase. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, United States) according to the manufacturer's instructions. The primers used in the experiments are shown in Table 1, and PCR was performed with a C1000 Thermal Cycler (Bio-Rad Laboratories Co., Ltd., CA, United States).

Protein expression analysis

Protein expression levels were analyzed by Western blotting according to standard protocols, and photographs were captured using a Bio-Rad molecular Imager[®] Gel DocTM XR+ imaging system with Image Lab[™] software. The primary antibodies against Hes1 (1:1000), RBP-J κ (1:1000), and β -actin (1:3000) and the appropriate secondary antibodies coupled with horseradish peroxidase (Bio-Rad, Munchen, Germany) were used to label the protein bands.

3D biliary epithelial cell culture

IBECs were purchased from ScienCell (Cat. Number 5100). Epithelial Cell Medium (EpiCM, ScienCell, Carlsbad, CA, United States. 4101) and additional Epithelial Cell Growth Supplement (EpiCGS, ScienCell 4152) were used for the maintenance and growth of IBECs. The medium used for the differentiation of cells was based on Kubota's Hepatoblast Growth Medium (PhoenixSongs Biologicals, Inc. Branford, CT, United States. 06405 11002-250). For IBEC differentiation, the medium was supplemented with HGF (10 ng/mL) and VEGF (20 ng/mL) (PeproTech Asia, Rehovot, Israel). The identification of the cell type was performed by

immunofluorescence staining using the following 3 antibodies: AFP (Abcam, GR201677-8, 1:200) for hepatocytes and CK19 (Santa Cruz Biotechnology, K2613, 1:200) and γ -GT (Abcam, GR304453-1, 1:300) for BECs. Goat anti-mouse Alexa Fluor[®] 488 secondary antibody (Life Technologies, 1613346) and DAPI (KeyGen Biotech. Co., Ltd., Nanjing, China. KGR0001) were used to visualize antibody staining and the nuclei of the cells.

For the formation of duct-like structures of IBECs in three-dimensional culture, a 4:6 (v/v) gel mixture of BD Matrigel Matrix Growth Factor Reduced High Concentration (BD, 356230) and collagen Type I Rat Tail (CORNING, 354236) was used. Cells (4×10^4) were seeded on the gel using a 15-well μ -Plate Angiogenesis plate (ibidi GmbH, Martinsried, Germany, 81506) and cultured in Kubota's hepatoblast differentiation medium for seven days. The immunofluorescence staining described above was used to show the structure and differentiation statuses.

Transfection assay

To study the interruption of gene expression, such as interruption of the Notch signaling pathway target molecule Hes1, in the IBEC 3D cell culture, Hes1 siRNA RNA and control siRNA purchased from Santa Cruz Biotechnology, Inc., were used in the cell culture and transfected into cells using Lipofectamine[®] RNAiMAX Reagent (Life Technologies, 13778) following the manufacturer's instructions.

Statistical analysis

The data are presented as the mean \pm SEM when repeated measurements were used and as the mean \pm SD when individual values were used. The statistical analysis was performed by the Mann-Whitney test when two sets of data were compared and by the Kruskal-Wallis test when more than three sets of data were compared. The statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, United States) with *P* values < 0.05 taken as

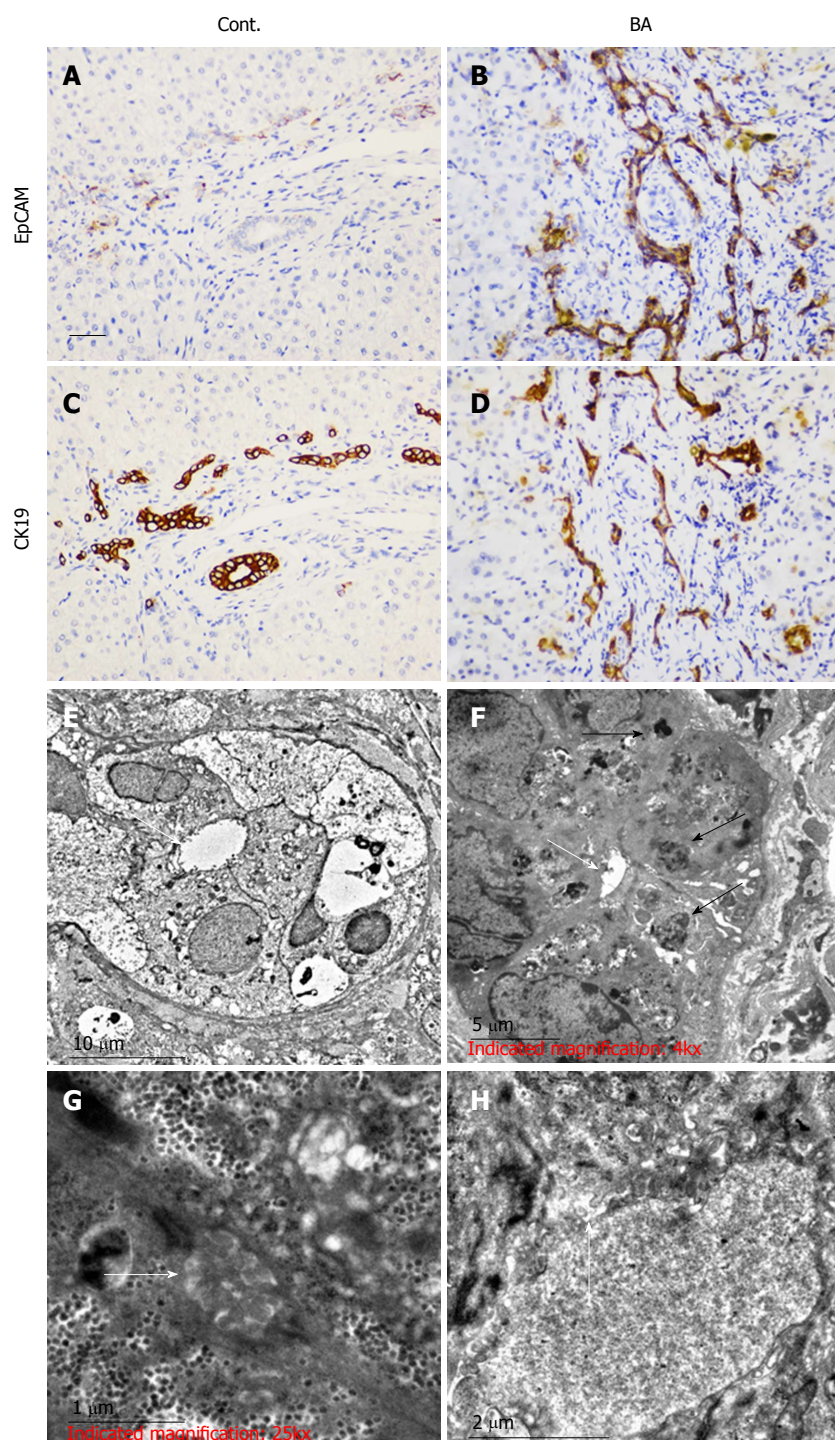


Figure 1 Expression levels of EpCAM and CK19 in neonatal liver biopsies and electron microscopy analysis of hepatic ultrastructure. To test the biliary epithelial marker expression levels, immunohistochemical staining was performed on disease control samples (Cont., choledochal cyst, A and C) and biliary atresia patient samples (BA, B and D). For the colocalization study of EpCAM and CK19, adjacent tissue sections were used. The scale bar is shown as 50 μ m. For the EM analysis, liver tissues from the Cont. (E and F) and BA patients (G and H) were sectioned at 50-70 nm and stained with uranyl acetate and lead citrate. Photographs of the bile capillary (E and G) and microvilli (F and H) were captured and presented. The scale bar is also shown in the photographs. $n = 5$ each for the CC and BA groups.

the level of significance.

RESULTS

Increase in EpCAM immunopositive cells in the liver tissue of BA patients and the study of the ultrastructure of cells

We used adjacent, age-matched sections for studying

immature BECs (EpCAM⁺) and immunohistochemical staining of the BEC marker (CK19⁺). Our results showed that compared with choledochal cysts as a control (Cont., Figure 1A and C) there were highly increased numbers of EpCAM⁺ cells in BA (Figure 1B and D). Most EpCAM⁺ cells were also CK19⁺, indicating that EpCAM is expressed in BECs. In choledochal cysts, disappearance

of the EpCAM⁺ marker indicated the mature status of the cells. Additionally, the bile ductules could be easily observed in choledochal cysts but not in BA.

The ultrastructural changes in the morphology of the bile ducts in BA patients were compared to those in CC patients. The results revealed a similar hepatic cellular ultrastructure appearance in both cases. Mild swelling of liver cells was observed with no obvious abnormalities in the nuclei, rough endoplasmic reticulum, or smooth endoplasmic reticulum, apart from slight hyperplasia. However, in the portal triad, abnormal narrowing of the bile capillary was observed together with irregular sediments of bilirubin particles in the BA group (Figure 1F) in contrast to the CC group (Figure 1E). Fewer microvilli and less bile salt deposition were found in the BA patient tissue sections (Figure 1H) than in the CC patient tissue sections (Figure 1G). Taken together, these data suggest that BECs in BA patients are not fully mature, which might affect bile transport.

Optimization of the dose of rhesus rotavirus for the induction of BA in mice

Viral infection is proposed to be a causative factor in the development of BA, and the RRV-induced BA mouse model is used to mimic the pathophysiological processes in human BA. However, high doses of virus (1×10^6 PFU/mL) often cause the early death of mice at days 5–7 after viral inoculation. To reduce mortality, we used a smaller dose of RRV (4×10^4 PFU/mL) to induce BA. After viral inoculation, the mice exhibited BA syndrome with jaundice (Figure 2A) and extrahepatic bile duct occlusion (Figure 2B). The survival rate with this dosage was 63.03% and significantly differed from that of the control group (supernatant without virus infection) (Figure 2C; $P < 0.05$). Changes in weight loss were significant, with the control group exhibiting a $10.0 \text{ g} \pm 0.25 \text{ g}$ change ($n = 15$) but the RRV-treated group only exhibiting a $5.6 \text{ g} \pm 1.3 \text{ g}$ change ($n = 26$) (Figure 2D; $P < 0.001$). After dissection and tissue sectioning of the livers of the BA mice, HE staining revealed that, in contrast to the normal control group (Figure 2E), a disappearance of the common bile duct was observed in the BA group (Figure 2F). At the portal triads, enlarged cystic ducts and inflammatory cell accumulation were also present at day 14 after RRV inoculation, in contrast to normal control mice (Figure 2H vs Figure 2G).

Presence of EpCAM⁺ and CK19⁺ bile ducts in RRV-inoculated BA mice

To identify the bile ducts, immunohistochemical staining was performed for both EpCAM and CK19 at days 7 and 14 after RRV inoculation, and RRV-inoculated mice were compared with control mice. As shown in Figure 3, EpCAM⁺ bile ducts were noted at both days 7 and 14 in the control mice (Figure 3A and 3C), but few EpCAM⁺ cells were found in the RRV-inoculated mouse BA livers at day 7 (Figure 3B). At day 14, more EpCAM⁺ cells were detected, even in the areas of the portal tracts that

were infiltrated with inflammatory cells. However, none of these cells were organized into ductule-like structures (Figure 3D). Similar to EpCAM, CK19⁺ ductules were observed in the control livers at days 7 and 14, although the signal was slightly weaker on day 7 (Figure 3E and G). However, in BA mice, CK19⁺ cells were found in the portal areas, but no ductule structures were observed (Figure 3F and H). This observation suggests the presence of BECs, especially at day 14 after RRV inoculation. However, their development, especially in the formation of the bile duct process, was interrupted.

Expression of Notch signaling pathway components in the liver tissues of RRV-inoculated BA mice

The Notch signaling pathway plays a key role in bile duct system development. To examine its function in this model, qPCR was employed to quantify the expression levels of key genes in the Notch signaling pathway. The results showed that at day 7, there was a marked reduction in the expression levels of Notch ligands [Jag1 and Delta-like (Dll1)], Notch receptors (Notch1 and Notch2), and the Notch target gene Hes1 in the BA liver tissues compared with control liver tissues (Figure 4A, $P < 0.05$ for Jag1 and Notch1, $P < 0.01$ for Hes1 compared with the control). At day 14, no obvious differences in the expression levels of Jag1, Dll1, Notch1 and Notch2 were observed between the control and BA groups, and a slight increase in the expression of Notch2 was even found, although not statistically significant ($P = 0.1434$). However, the levels of Hes1 transcripts remained significantly reduced (10-fold compared with the control; $P < 0.001$; Figure 4B). The protein expression levels were then examined using Western blotting, which yielded similar results, namely, that the concentration of Hes1 protein in the mouse BA group was significantly lower on both day 7 and day 14 after RRV inoculation than that in the control group (Figure 4C). These data suggest that BA in RRV-inoculated mice may be caused, at least partially, by the downregulation of Hes1 expression.

Downregulation of RBP-J κ protein in the liver tissues of BA mice

As we observed reduced expression of Hes1, we then chose to investigate the expression levels of the regulators of Hes1. We determined the expression of the cotranscriptional factor RBP-J κ by Western blotting, and the results demonstrated that there was a minimal expression of RBP-J κ at both day 7 and day 14 in the BA mice compared to controls (Figure 4C). This finding suggests that downregulation of RBP-J κ may be the cause of the reduced expression of Hes1 and may subsequently hinder normal bile duct development.

Hes1 siRNA in the development of duct-like structures in 3D cell culture

The function of Hes1 in bile duct development was evaluated using a Hes1 siRNA transfection assay in a BEC

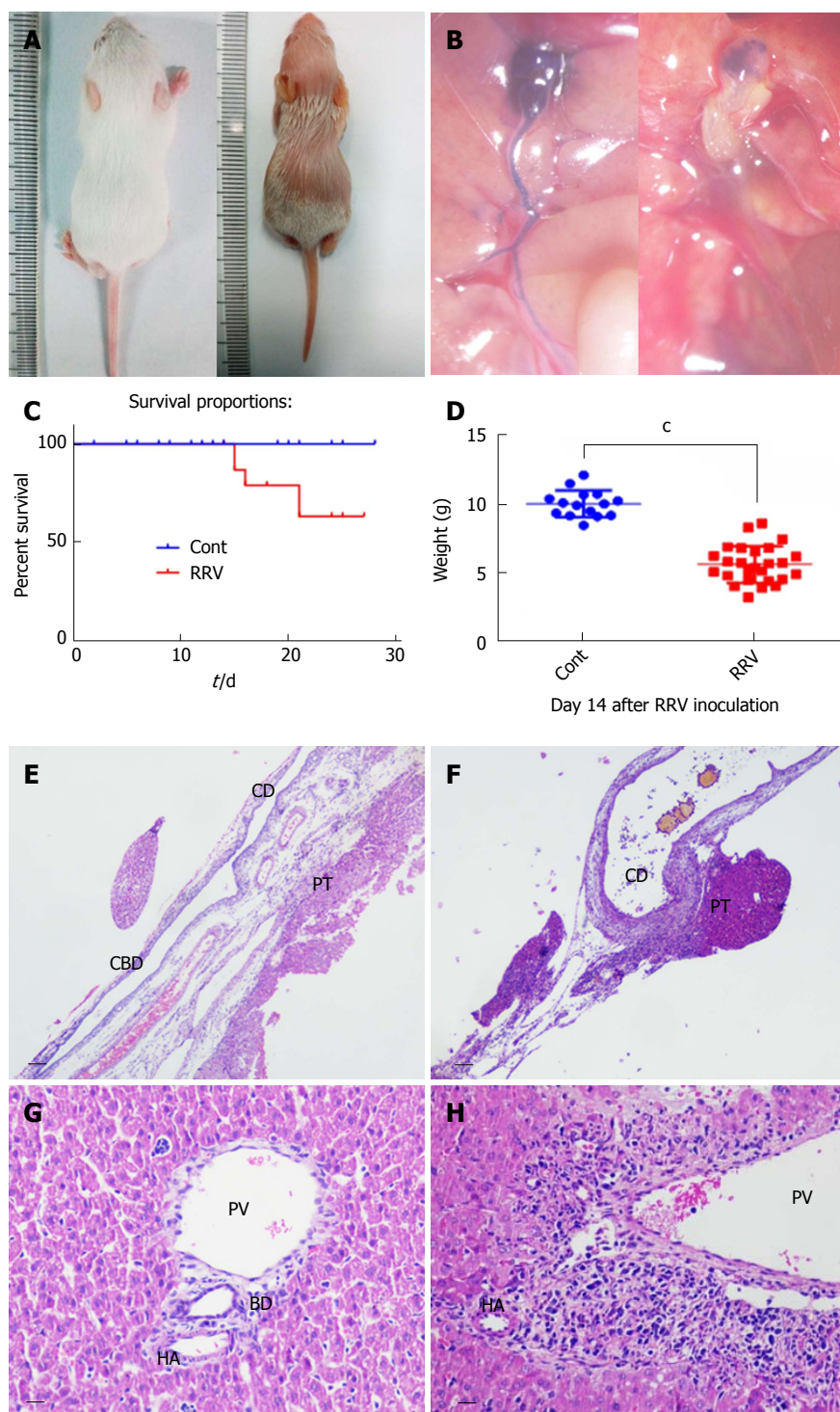


Figure 2 Biliary atresia in neonatal mice with a low dose of rhesus rotavirus inoculation. To study the expression levels of the components of the Notch signaling pathway in the biliary atresia (BA) animal model, a lower dose of 20 μ L of rhesus rotavirus (RRV) with a titer of 4×10^4 PFU/mL was used. The morphology and extrahepatic block were photographed (A and B). The survival rate was measured at 21 d after viral inoculation (C). BA syndrome was observed between days 5 and 6. The body weights of the mice were measured at day 14 and compared with that of the control group (injected with culture supernatant but no virus) (D) ($P < 0.001$, $n = 15$ in the Cont. group, and $n = 26$ in the RRV group). After dissection, the portal triad (E and F) and liver tissue at day 14 (G and H) were sectioned, and histological analysis was performed. The scale bar is shown as 0.2 mm in the portal triad sections and as 50 μ m in the liver tissue sections. CD: Cystic duct; CBD: Common bile duct; PT: Portal triad; PV: Portal vein; HA: Hepatic artery; BD: Bile duct.

culture. When BECs were cultured in growth medium, immunostaining showed that the cells expressed positive signals for CK19 and γ -GT but also a positive signal for AFP, which suggests the immature status of the cells (Figure 5, N. Medium). However, when the cells were

cultured in matrix gel + collagen gel (the mixed gel), the cells showed positive signals only for the BEC markers CK19 and γ -GT (Figure 5, Cont.), which suggested that the maturation process was underway. The formation of cells into duct-like structures was also observed

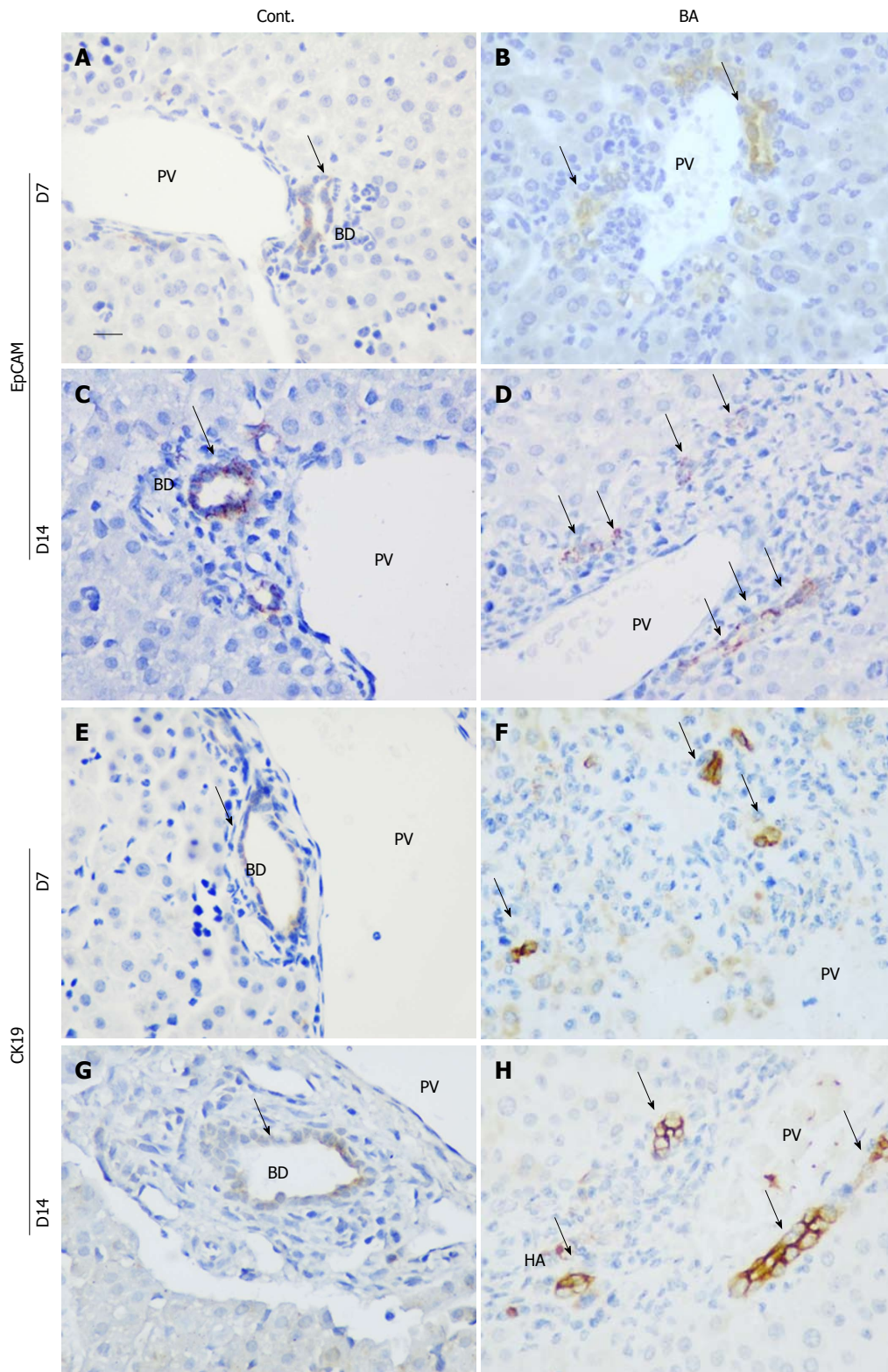


Figure 3 Expression levels of EpCAM and CK19 in the liver and ultrastructure of biliary epithelial cells. The expression levels of EpCAM and CK19 were analyzed using immunohistochemical staining with antibodies. The tissue sections were collected at day 7 (A, B, E and F) and day 14 (C, D, G and H). After they were dewaxed, the sections were stained with EpCAM and CK19 antibodies, and a comparison of the mice in the control group (Cont.) and those in the rhesus rotavirus inoculation group was performed. The scale bar shown is 100 μ m. The black arrow indicates the positive signal. BA: Biliary atresia; CD: Cystic duct; CBD: Common bile duct; PT: Portal triad; PV: Portal vein; HA: Hepatic artery; BD: Bile duct.

after seven days in cell culture (Figure 5, Cont.). An enhanced density of duct-like structures was observed with prolonged culture time. These data indicated that

the culture conditions promoted the maturation of cells. The low toxicity of the transfection reagent for duct-like structure formation was tested, and the results showed

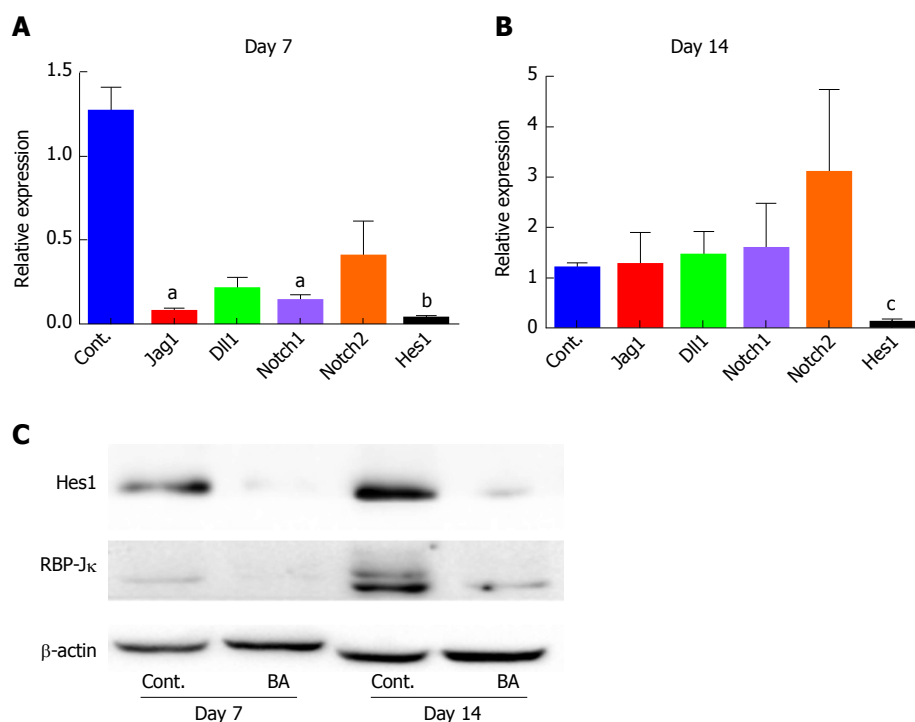


Figure 4 Expression levels of Notch signaling components in the liver of rhesus rotavirus-inoculated mice. The mRNA levels of Notch signaling components, including Jag1, Dll1, Notch1, Notch2 and Hes1, were detected using quantitative real-time PCR with appropriate primers at day 7 and day 14 after RRV inoculation. A: The relative expression levels compared with the normal control are shown for day 7 (^a $P < 0.05$; ^b $P < 0.01$; $n = 6$ in the control group and $n = 10$ in the RRV group); B: day 14 (^c $P < 0.001$); C: The levels of the Hes1 protein and the expression of the transcriptional coregulator RBP-J κ were detected by Western blot ($n = 2$ or 3 in each group; the proteins were mixed and loaded).

that there was no obvious difference in duct formation in the transfection reagent group (Figure 5, Lipo) compared with the control group. However, Hes1 siRNA transfection induced an obvious difference compared with the irrelevant siRNA control (Figure 5, Hes1 siRNA vs Cont. siRNA), as no duct-like structures were observed in Hes1 siRNA-transfected cells, and these cells were kept in an immature state according to AFP⁺ staining.

DISCUSSION

EpCAM is a cell surface-expressed transmembrane molecule that is present not only on epithelial cells but also on a variety of cells, including stem cells and cancer stem cells^[19]. Using a BEC marker for colocalization, CK19, we noted that the number of EpCAM⁺ cells was increased in BA patients but not in CC control patients, suggesting active proliferation of BECs in BA. Furthermore, CK19 has been reported as a marker for ductal reactivity in tumor studies^[20,21]. EpCAM expression in epithelial cells has been linked to the epithelial-mesenchymal transition (EMT)^[22], but its role in this process remains controversial^[23]. Both EMT and the ductal reaction are relevant to the development of tissue fibrosis^[24], although the associated mechanisms have not been fully defined^[25]. Elucidating EpCAM expression and its regulation in BA patients and/or experimental models of BA might provide new information regarding

the relationship of the ductal reaction with tissue fibrosis, especially as some previous studies have already reported the occurrence of EMT in BA patients^[26-28].

We found that EpCAM⁺ cell number was closely correlated with the expression of the Notch signaling target gene Hes1 and that Hes1 expression was increased in the nuclei of BECs in BA patients and RRV-inoculated mice. The expression of Hes1, as a nuclear regulatory protein, has been well-studied in biliary tract development, and as such, the Hes1-null mice showed a relatively normal ductal plate consisting of cytokeratin-positive and DBA-positive cholangiocyte precursors. However, by postnatal day 0, these mice presented with gallbladder agenesis and severe hypoplasia of the extrahepatic bile ducts^[12], suggesting immature differentiation of BECs. In accordance with previous observations, our data of the 3D BEC cell culture also demonstrate that low levels of Hes1 prevented the formation of duct-like structures. Additionally, the increase in the expression levels of the mature BEC markers γ -GT and CK19 and the reduction in the expression of AFP are further evidence that Hes1 functions in the maturation and structural reorganization of these cells. We sought to examine the expression of Hes1 in human BA samples. Immunohistochemistry staining revealed heterogeneity in the results, even among the same tissue samples (data not shown). Thus, the Hes1 effect in BA might be related to different stages (inflammation or fibrogenesis) of the

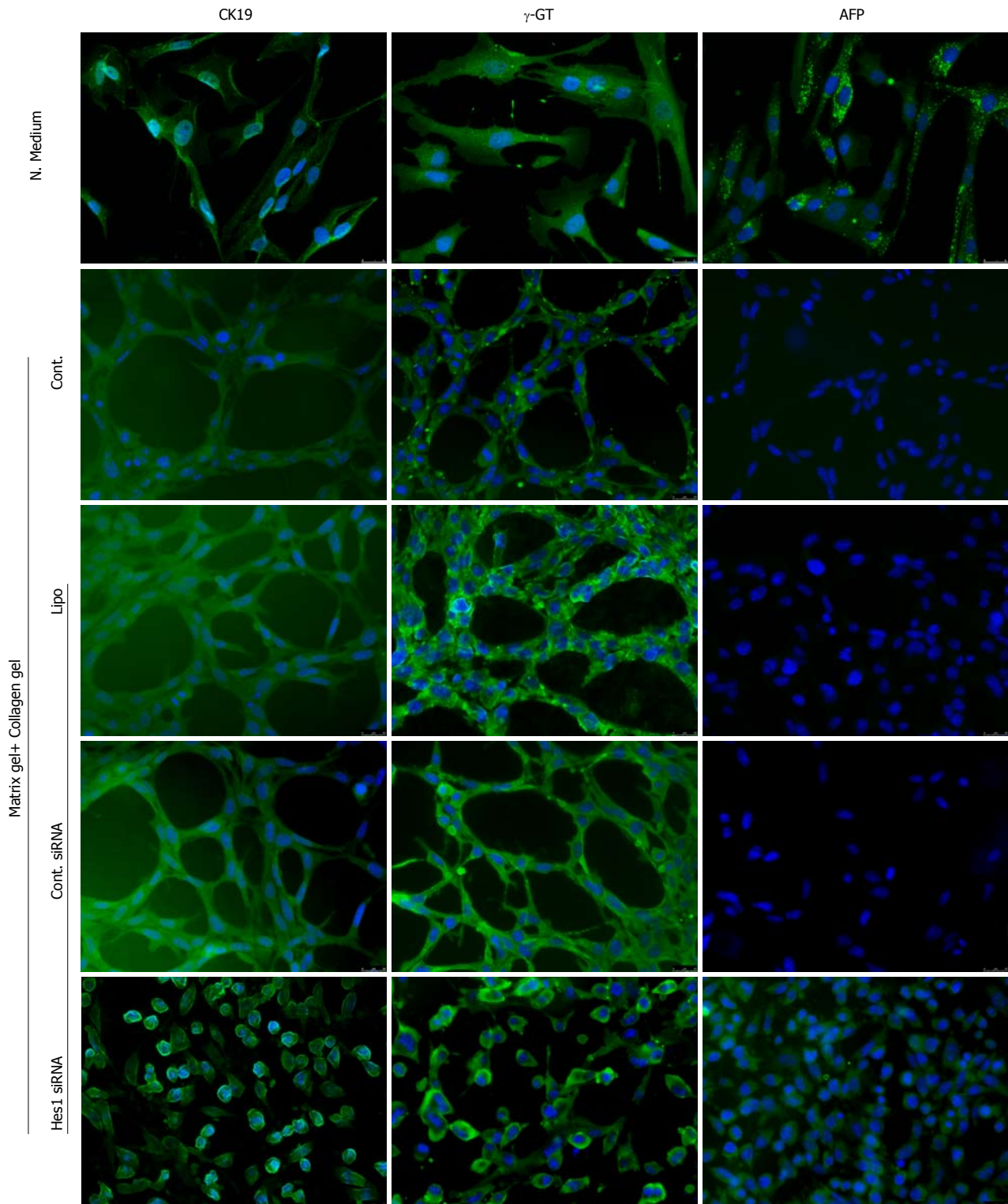


Figure 5 Hes1 siRNA in 3D biliary epithelial cell culture. Biliary epithelial cells (BECs) were cultured in either normal growth medium (N. medium) or in matrix + collagen mixed gel (detailed components are given in the Materials and Methods section). The cells were cultured without a transfection reagent (Cont.), with Lipofectamine (Lipo) only, with irrelevant control siRNA (Cont. siRNA) or with Hes1 siRNA for seven days, then fixed and stained with anti-CK19, γ -GT and AFP antibodies, and finally counter-stained with Alexa Fluor[®]488. Photographs of the cells were taken with a Leica DMI8 inverted fluorescence microscope, and the groups were then compared. The experiment was duplicated, and one set of representative results is shown here.

disease, as most of the BA patients in the clinic were in the stage of tissue fibrosis or later stages of the disease. Recruiting earlier-stage patients in whom inflammation is still progressing will help to clarify the alterations in Hes1

expression in BA patients.

The expression of Hes1 is under the control of coregulatory factors, particularly NICD and RBP-J κ . NICD is a component of the Notch signaling pathway, and in our

study, its expression was relatively normal, although some intracellular signaling pathway components, such as gamma secretase, might still affect the levels of NICD^[29]. Here, we provide evidence of a clear decline in the expression of RBP-J κ , which might be the cause of reduced Hes1. A similar phenomenon was observed in RBP-J κ KO mice. In those animals, the mature ducts were significantly reduced with respect to similarly treated wild-type mice^[30]. It has been reported that RBP-J κ expression is modulated by many regulatory factors, including viral infection^[31,32]. In Epstein-Barr virus (EBV) infection, the nuclear antigens (EBNA) 3A and 3C can compete with EBNA 2 in binding with RBP-J κ , thus inhibiting RBP-J κ function. This diminishes the functional activity of Notch signaling by reducing the expression of Hes1^[33]. However, whether or not there is any similarity between the EBV and RRV components in Hes1 expression remains to be investigated. At the end of the study, a high dose of Pentobarbital was administered to the mice, and then samples were collected for analysis. It has been shown that Pentobarbital affects bile secretion^[34], however, as we used a control group, and the comparative results showed a difference between the treatment and control groups, the effect of Pentobarbital might not have directly influenced the results. Further research with mouse sacrifice by dislocation of the cervical spine will help to clarify this point.

The results of this study suggest that the reduced Hes1 expression may have contributed to reformation of the functional bile duct, but other factors cannot be excluded, such as inflammation or viral infection. The construction or adaptation of conditional knockout mice with targeted Hes1 knockdown on day 7, together with the BA mouse model with Hes1 overexpression, might help to confirm that Hes1 is the only contributing factor in this BA mouse model. The results of these experiments will provide more information regarding whether Hes1 overexpression has any therapeutic effect in the treatment of BA.

The mouse model used in this study represents acute viral infection-induced biliary atresia, in which tissue fibrosis is absent. The direct comparison of the mouse data with the patient data is not appropriate, as in patients, substantial tissue fibrosis is often observed at the later stage. Therefore, the development of a chronic BA mouse model will better mimic the condition observed in BA patients, allowing for a more accurate comparison of Hes1 data.

In conclusion, our study demonstrated that EpCAM⁺ cells are increased in the liver tissue of BA patients and the BA mouse model. The experimental results presented here suggest that the Notch signaling effector gene Hes1 might contribute to the disease processes in BA and that downregulation of RBP-J κ might account for the pathophysiological changes in the bile ducts that were observed in the BA mouse model. The 3D BEC culture further confirmed the effect of Hes1 on the

maturation and the duct-like structural formation of cells, which helps to further explain the dysregulation of structure formation in BA patients.

ARTICLE HIGHLIGHTS

Research background

An increased number of immature biliary epithelial cells and distorted bile ductules were observed in biliary atresia (BA), but the causes of these changes are unknown. The Notch signaling pathway is related to the development and differentiation of biliary epithelial cells. The target gene Hes1 is essential for tubular formation and maintenance. However, the effect of altered Hes1 expression in biliary atresia has not been established.

Research motivation

Notch signaling is one of the main pathways involved in bile duct development. However, its function in BA is not well known. Analysis of Notch signaling molecules using an established BA animal model, and 3D cell culture system might provide novel insights into the pathogenesis of BA.

Research objectives

The expression of Notch signaling pathway-related molecules was detected in a BA mouse model. The function of Hes1 downregulation was further examined using a 3D cell culture system. The results of this study can be expanded upon in future research of human BA patients by examining Hes1 expression and its relationship with BA pathogenesis.

Research methods

Immature biliary epithelial cells and bile duct structure distortion were examined in BA patients and in a BA mouse model. The expression of Notch signaling pathway-related molecules was detected in the mouse model by qPCR, and the expression of Hes1 and its gene regulatory protein was further confirmed by Western blotting. Finally, in 3D cell culture, the effects of Hes1 inhibition induced by siRNA transfection on duct-like structure formation were observed.

Research results

The results revealed the presence of immature biliary epithelial cells and distorted structures in both the BA patients and animal model. The downregulation of Hes1 expression, together with its transcriptional co-regulator RBP-J κ , was observed in the BA mouse model. The siRNA-mediated inhibition of Hes1 completely blocked duct-like structure formation in the 3D cell culture system. However, Hes1 expression in BA patients must be further evaluated to confirm its function in the disease process.

Research conclusions

In conclusion, the results of the current study indicate that the immature biliary epithelial cells and defective duct-like structure formation in BA might be partly related to downregulation of the expression of the Notch signaling target gene Hes1. The use of a 3D epithelial cell culture system might help to identify other potential molecules, including those involved in epithelial cell maturation and duct-like structure formation.

Research perspectives

The potential effects of Hes1 observed in the BA mouse model and cell culture involving biliary epithelial cell maturation and duct-like structure formation suggest that Hes1 might contribute to the pathogenesis of BA. However, further examination of BA patient samples is necessary to better understand the role of Hes1 in the BA disease process.

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REFERENCES

- Hartley JL, Davenport M, Kelly DA. Biliary atresia. *Lancet* 2009; **374**: 1704-1713 [PMID: 19914515 DOI: 10.1016/S0140-6736(09)60946-6]
- Yoon PW, Bresee JS, Olney RS, James LM, Khoury MJ. Epidemiology of biliary atresia: a population-based study. *Pediatrics* 1997; **99**: 376-382 [PMID: 9041292 DOI: 10.1542/peds.99.3.376]
- Davenport M. Biliary atresia. *Semin Pediatr Surg* 2005; **14**: 42-48 [PMID: 15770587 DOI: 10.1053/j.sempedsurg.2004.10.024]
- Gallo A, Esquivel CO. Current options for management of biliary atresia. *Pediatr Transplant* 2013; **17**: 95-98 [PMID: 23347466 DOI: 10.1111/ptr.12040]
- Bessho K, Bezerra JA. Biliary atresia: will blocking inflammation tame the disease? *Annu Rev Med* 2011; **62**: 171-185 [PMID: 21226614 DOI: 10.1146/annurev-med-042909-093734]
- Boulter L, Lu WY, Forbes SJ. Differentiation of progenitors in the liver: a matter of local choice. *J Clin Invest* 2013; **123**: 1867-1873 [PMID: 23635784 DOI: 10.1172/JCI66026]
- Morell CM, Strazzabosco M. Notch signaling and new therapeutic options in liver disease. *J Hepatol* 2014; **60**: 885-890 [PMID: 24308992 DOI: 10.1016/j.jhep.2013.11.028]
- Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, Qi M, Trask BJ, Kuo WL, Cochran J, Costa T, Pierpont ME, Rand EB, Piccoli DA, Hood L, Spinner NB. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet* 1997; **16**: 243-251 [PMID: 9207788 DOI: 10.1038/ng0797-243]
- Oda T, Elkhouloun AG, Pike BL, Okajima K, Krantz ID, Genin A, Piccoli DA, Meltzer PS, Spinner NB, Collins FS, Chandrasekharappa SC. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat Genet* 1997; **16**: 235-242 [PMID: 9207787 DOI: 10.1038/ng0797-235]
- McCright B, Lozier J, Gridley T. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. *Development* 2002; **129**: 1075-1082 [PMID: 11861489]
- Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T. The role of notch signaling in the development of intrahepatic bile ducts. *Gastroenterology* 2004; **127**: 1775-1786 [PMID: 15578515 DOI: 10.1053/j.gastro.2004.09.004]
- Sumazaki R, Shiojiri N, Itoyama S, Masu M, Keino-Masu K, Osawa M, Nakauchi H, Kageyama R, Matsui A. Conversion of biliary system to pancreatic tissue in Hes1-deficient mice. *Nat Genet* 2004; **36**: 83-87 [PMID: 14702043 DOI: 10.1038/ng1273]
- Zhang RZ, Yu JK, Peng J, Wang FH, Liu HY, Lui VC, Nicholls JM, Tam PK, Lamb JR, Chen Y, Xia HM. Role of CD56-expressing immature biliary epithelial cells in biliary atresia. *World J Gastroenterol* 2016; **22**: 2545-2557 [PMID: 26937142 DOI: 10.3748/wjg.v22.i8.2545]
- de Boer CJ, van Krieken JH, Janssen-van Rhijn CM, Litvinov SV. Expression of Ep-CAM in normal, regenerating, metaplastic, and neoplastic liver. *J Pathol* 1999; **188**: 201-206 [PMID: 10398165 DOI: 10.1002/(SICI)1096-9896(199906)188:2<201::AID-PATH339>3.0.CO;2-8]
- Joplin R, Kachilele S. Human intrahepatic biliary epithelial cell lineages: studies in vitro. *Hepatocyte Transplantation*: Springer, 2009: 193-206
- Sekiya S, Suzuki A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. *J Clin Invest* 2012; **122**: 3914-3918 [PMID: 23023701 DOI: 10.1172/JCI63065]
- Stamp L, Crosby HA, Hawes SM, Strain AJ, Pera MF. A novel cell-surface marker found on human embryonic hepatoblasts and a subpopulation of hepatic biliary epithelial cells. *Stem Cells* 2005; **23**: 103-112 [PMID: 15625127 DOI: 10.1634/stemcells.2004-0147]
- Arnold M, Patton JT, McDonald SM. Culturing, storage, and quantification of rotaviruses. *Curr Protoc Microbiol* 2009; **15**: Unit 15C.3 [PMID: 19885940 DOI: 10.1002/9780471729259.mc15c03s15]
- Dollé L, Theise ND, Schmelzer E, Boulter L, Gires O, van Grunsven LA. EpCAM and the biology of hepatic stem/progenitor cells. *Am J Physiol Gastrointest Liver Physiol* 2015; **308**: G233-G250 [PMID: 25477371 DOI: 10.1152/ajpgi.00069.2014]
- Yoon SM, Gerasimidou D, Kuwahara R, Hytioglou P, Yoo JE, Park YN, Theise ND. Epithelial cell adhesion molecule (EpCAM) marks hepatocytes newly derived from stem/progenitor cells in humans. *Hepatology* 2011; **53**: 964-973 [PMID: 21319194 DOI: 10.1002/hep.24122]
- Zhang Q, Zhang CS, Xin Q, Ma Z, Liu GQ, Liu BB, Wang FM, Gao YT, Du Z. Perinodular ductular reaction/epithelial cell adhesion molecule loss in small hepatic nodules. *World J Gastroenterol* 2014; **20**: 10908-10915 [PMID: 25152593 DOI: 10.3748/wjg.v20.i31.10908]
- Gao J, Yan Q, Wang J, Liu S, Yang X. Epithelial-to-mesenchymal transition induced by TGF- β 1 is mediated by AP1-dependent EpCAM expression in MCF-7 cells. *J Cell Physiol* 2015; **230**: 775-782 [PMID: 25205054 DOI: 10.1002/jcp.24802]
- Massoner P, Thomm T, Mack B, Untergasser G, Martowicz A, Bobowski K, Klocker H, Gires O, Pühr M. EpCAM is overexpressed in local and metastatic prostate cancer, suppressed by chemotherapy and modulated by MET-associated miRNA-200c/205. *Br J Cancer* 2014; **111**: 955-964 [PMID: 24992580 DOI: 10.1038/bjc.2014.366]
- Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 2003; **112**: 1776-1784 [PMID: 14679171 DOI: 10.1172/JCI20530]
- Williams MJ, Clouston AD, Forbes SJ. Links between hepatic fibrosis, ductular reaction, and progenitor cell expansion. *Gastroenterology* 2014; **146**: 349-356 [PMID: 24315991 DOI: 10.1053/j.gastro.2013.11.034]
- Omenetti A, Bass LM, Anders RA, Clemente MG, Francis H, Guy CD, McCall S, Choi SS, Alpini G, Schwarz KB, Diehl AM, Whittington PF. Hedgehog activity, epithelial-mesenchymal transitions, and biliary dysmorphogenesis in biliary atresia. *Hepatology* 2011; **53**: 1246-1258 [PMID: 21480329 DOI: 10.1002/hep.24156]
- Xiao Y, Zhou Y, Chen Y, Zhou K, Wen J, Wang Y, Wang J, Cai W. The expression of epithelial-mesenchymal transition-related proteins in biliary epithelial cells is associated with liver fibrosis in biliary atresia. *Pediatr Res* 2015; **77**: 310-315 [PMID: 25406900 DOI: 10.1038/pr.2014.181]
- Deng YH, Pu CL, Li YC, Zhu J, Xiang C, Zhang MM, Guo CB. Analysis of biliary epithelial-mesenchymal transition in portal tract fibrogenesis in biliary atresia. *Dig Dis Sci* 2011; **56**: 731-740 [PMID: 20725787 DOI: 10.1007/s10620-010-1347-6]
- Roncarati R, Sestan N, Scheinfeld MH, Berechid BE, Lopez PA, Meucci O, McGlade JC, Rakic P, D'Adamio L. The gamma-secretase-generated intracellular domain of beta-amyloid precursor protein binds Numb and inhibits Notch signaling. *Proc Natl Acad Sci U S A* 2002; **99**: 7102-7107 [PMID: 12011466 DOI: 10.1073/pnas.102192599]
- Fiorotto R, Raizner A, Morell CM, Torsello B, Scirpo R, Fabris L, Spirli C, Strazzabosco M. Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice. *J Hepatol* 2013; **59**: 124-130 [PMID: 23500150 DOI: 10.1016/j.jhep.2013.02.025]
- Kohn A, Dong Y, Mirando AJ, Jesse AM, Honjo T, Zuscik MJ, O'Keefe RJ, Hilton MJ. Cartilage-specific RBPjk-dependent and -independent Notch signals regulate cartilage and bone development. *Development* 2012; **139**: 1198-1212 [PMID: 22354840 DOI: 10.1242/dev.070649]
- Zhang L, Zhu C, Guo Y, Wei F, Lu J, Qin J, Banerjee S, Wang J, Shang H, Verma SC, Yuan Z, Robertson ES, Cai Q. Inhibition of KAP1 enhances hypoxia-induced Kaposi's sarcoma-associated herpesvirus reactivation through RBP-Jc. *J Virol* 2014; **88**:

- 6873-6884 [PMID: 24696491 DOI: 10.1128/JVI.00283-14]
- 33 **Hsieh JJ**, Nofziger DE, Weinmaster G, Hayward SD. Epstein-Barr virus immortalization: Notch2 interacts with CBF1 and blocks differentiation. *J Virol* 1997; **71**: 1938-1945 [PMID: 9032325]
- 34 **Mills CO**, Freeman JF, Salt PJ, Elias E. Effect of anaesthetic agents on bile flow and biliary excretion of ¹³¹I-cholylglycyltyrosine in the rat. *Br J Anaesth* 1989; **62**: 311-315 [PMID: 2784686 DOI: 10.1093/bja/62.3.311]

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Basic Study

High expression of type I inositol 1,4,5-trisphosphate receptor in the kidney of rats with hepatorenal syndrome

Jing-Bo Wang, Ye Gu, Ming-Xiang Zhang, Shun Yang, Yan Wang, Wei Wang, Xi-Ran Li, Yi-Tong Zhao, Hai-Tao Wang

Jing-Bo Wang, Ye Gu, Ming-Xiang Zhang, Yan Wang, Wei Wang, Xi-Ran Li, Yi-Tong Zhao, Liver Cirrhosis Ward, the Sixth People's Hospital of Shenyang, Shenyang 110006, Liaoning Province, China

Shun Yang, Liaoning Cancer Hospital & Institute, Shenyang 110042, Liaoning Province, China

Hai-Tao Wang, Department of General Surgery, the Second Affiliated Hospital of Shenyang Medical College, Shenyang 110002, Liaoning Province, China

ORCID number: Jing-Bo Wang (0000-0001-9207-6245); Ye Gu (0000-0002-3798-1119); Ming-Xiang Zhang (0000-0001-6519-3497); Shun Yang (0000-0003-4743-0588); Yan Wang (0000-0002-0427-3458); Wei Wang (0000-0002-4964-3280); Xi-Ran Li (0000-0002-3253-407X); Yi-Tong Zhao (0000-0002-5059-8608); Hai-Tao Wang (0000-0002-7385-0642).

Author contributions: Wang JB, Wang HT and Gu Y contributed equally to this work, and all of them were involved in the design and performing of the experiment, data analysis and drafting of the article; Zhang MX, Wang Y and Yang S participated in the study, hepatic and renal pathological examination and biochemical test; Li XR, Wang W and Zhao YT completed data analysis, Western blot analysis and real-time PCR and drafting of the paper.

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Correspondence to: Hai-Tao Wang, MSc, Surgeon, Department of General Surgery, the Second Affiliated Hospital of Shenyang Medical College, No. 20, Beijiuma Road, Heping District, Shenyang 110002, Liaoning Province, China. whszyypwk@163.com
Telephone: +86-18002452018
Fax: +86-24-31251510

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Abstract

AIM

To detect the expression of type I inositol 1,4,5-trisphosphate receptor (IP₃RI) in the kidney of rats with hepatorenal syndrome (HRS).

METHODS

One hundred and twenty-five Sprague-Dawley rats were randomly divided into four groups to receive an intravenous injection of D-galactosamine (D-GalN) plus lipopolysaccharide (LPS; group G/L, *n* = 50), D-GalN alone (group G, *n* = 25), LPS alone (group L, *n* = 25), and normal saline (group NS, *n* = 25), respectively.

At 3, 6, 9, 12, and 24 h after injection, blood, liver, and kidney samples were collected. Hematoxylin-eosin staining of liver tissue was performed to assess hepatocyte necrosis. Electron microscopy was used to observe ultrastructural changes in the kidney. Western blot analysis and real-time PCR were performed to detect the expression of IP₃RI protein and mRNA in the kidney, respectively.

RESULTS

Hepatocyte necrosis was aggravated gradually, which was most significant at 12 h after treatment with D-galactosamine/lipopolysaccharide, and was characterized by massive hepatocyte necrosis. At the same time, serum levels of biochemical indicators including liver and kidney function indexes were all significantly changed. The structure of the renal glomerulus and tubules was normal at all time points. Western blot analysis indicated that IP₃RI protein expression began to rise at 3 h ($P < 0.05$) and peaked at 12 h ($P < 0.01$). Real-time PCR demonstrated that IP₃RI mRNA expression began to rise at 3 h ($P < 0.05$) and peaked at 9 h ($P < 0.01$).

CONCLUSION

IP₃RI protein expression is increased in the kidney of HRS rats, and may be regulated at the transcriptional level.

Key words: Hepatorenal syndrome; Type I inositol 1,4,5-trisphosphate receptor; Glomerular mesangial cells; Vascular smooth muscle cells

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Core tip: Type I inositol 1,4,5-trisphosphate receptor (IP₃RI) protein expression is increased in the kidney of hepatorenal syndrome (HRS) rats, and IP₃RI protein expression may be regulated at the transcriptional level. Increased expression of IP₃RI may be closely associated with HRS development and progression through excessive renal vascular contraction resulting in insufficient renal blood perfusion.

Wang JB, Gu Y, Zhang MX, Yang S, Wang Y, Wang W, Li XR, Zhao YT, Wang HT. High expression of type I inositol 1,4,5-trisphosphate receptor in the kidney of rats with hepatorenal syndrome. *World J Gastroenterol* 2018; 24(29): 3273-3280 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i29/3273.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i29.3273>

INTRODUCTION

Hepatorenal syndrome (HRS), one of the most severe complications of liver failure (LF) and the leading cause of death in LF^[1], is functional renal failure secondary to

LF^[2-10]. At present, the exact pathogenesis of HRS is still unclear, and the reduction in renal blood flow induced by renal vasoconstriction is considered to play a central role in the development of HRS^[11,12]. Renal blood flow is regulated by the contraction and relaxation of vascular smooth muscle cells (VSMCs) of glomerular afferent arteries and glomerular mesangial cells (GMCs), while the contraction and relaxation of VSMCs and GMCs are regulated by intracellular Ca²⁺ concentrations^[13,14]. GMCs are in direct contact with glomerular endothelial cells. When GMCs contract, glomerular mesangial volume decreases by 20%-25%, glomerular capillary plexuses are reduced, and the area for glomerular filtration is reduced. Inositol 1,4,5-trisphosphate (IP₃) receptor (IP₃Rs) is the main Ca²⁺ release channel in cells. IP₃ is an intercellular second messenger mediating transmembrane signal transmission. When binding to IP₃Rs, IP₃ mediates intracellular calcium release and extracellular calcium influx^[15,16]. VSMCs and GMCs transmit extracellular signals into the cell via the IP₃-IP₃R pathway, increasing intracellular Ca²⁺ concentrations^[17,18]. Is high expression of renal IP₃Rs associated with HRS? To answer this question, in the present study we detected the expression of IP₃RI protein and mRNA in the kidney of HRS rats to determine the relationship between IP₃RI expression and HRS.

MATERIALS AND METHODS

Materials

Specific pathogen-free (SPF) Sprague-Dawley (SD) rats, weighing 220 g ± 20 g, were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences (Animal Certificate No. SCXK-2017-004; Beijing, China). Prior to experimentation, the rats were reared in separate cages at 23 °C ± 3 °C under a 12 h/12 h light/dark cycle, with free access to ordinary chow (purchased from the Laboratory Animal Center of China Medical University, Shenyang, China) and water. After one week of adaptation, the rats were used in the experiments.

D-galactosamine (D-GalN) and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO, United States). Anti-IP₃RI antibody was obtained from US Biological (St. Salem, OR, United States). An enhanced chemiluminescence (ECL) kit was purchased from Pierce, Dallas, TX, United States. RNAiso™ plus, Prime Script™ RT Reagent Kit, and SYBR® Premix EX Tag™ were purchased from TakaRa (Shiga, Japan).

Rat model of HRS

One hundred and twenty-five SD rats of SPF grade, weighing 220 ± 20 g, were randomly divided into four groups to receive an intravenous injection of D-GalN plus LPS (group G/L), D-GalN alone (group G), LPS alone (group L), and normal saline (group NS), respectively. Each group was further divided into five subgroups for testing at different time points (3, 6, 9,

12, and 24 h). Group G/L contained ten rats at each time point, and the other groups contained five rats at each time point. The rats were weighed and then injected with D-GalN (400 mg/kg body weight) and/or LPS (32 µg/kg) or NS (2 mL/kg) *via* the tail vein. Rats that died during the modeling process were excluded from the study. At 3, 6, 9, 12, and 24 h after modeling, the rats in groups G/L, G, and L were anesthetized with 0.8% pentobarbital sodium at 40 mg/kg *via* intraperitoneal injection and sacrificed to obtain liver and kidney tissues. A section of each tissue was fixed in formalin, and the remainder was preserved at -80 °C for Western blot and real-time PCR analysis of IP₃RI protein and mRNA expression, respectively.

Western blot analysis

For total protein preparation, renal tissue was lysed for 15 min in a lysis solution containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 5 mg/mL leupeptin, sodium orthovanadate, sodium fluoride, and 1 mmol/L PMSF, and then centrifuged at 12000 rpm for 12 min. The supernatant was collected and preserved at -80 °C.

After total protein concentration was determined using the bicinchoninic acid (BCA) method, the protein samples were mixed with 5 × loading buffer at a ratio of 4:1 (v/v), boiled for 5 min, resolved by 8% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The membranes were then blocked with 5% skimmed milk, Tris-buffered saline and Tween-20, and incubated with primary antibody against IP₃RI (dilution, 1:1000) at 4 °C overnight. This was followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (dilution, 1:3000) for 2 h at room temperature. The immunoblots were visualized using an enhanced chemiluminescence system. The molecular weight of the target band was 230 kDa. β-actin (45 kDa) was used as an internal control. Digital imaging software was used for densitometry analyses, and the relative IP₃RI level was calculated as IP₃RI grey value divided by β-actin grey value.

Real-time PCR

Total RNA was prepared from renal tissue using Trizol according to the manufacturer's instructions. After reverse transcription to cDNA in a 10-µL system containing 2.0 µL of 5 × Prime Script™ Buffer (for real time), 0.5 µL of Prime Script™ RT Enzyme Mi, 0.5 µL of Oligo dT Primer (50 µmol/L), 0.5 µL of Random 6-mers (100 µmol/L), 5.5 µL of RNase Free dH₂O, and 1.0 µL of RNA (500 ng/µL), real-time PCR was performed in a 25-µL system containing 12.5 µL of 2 × SYBR Premix Ex Tag™, 0.5 µL of PCR Forward Primer (10 µmol/L), 0.5 µL of PCR Reverse Primer (10 µmol/L), 9.5 µL of RNase Free dH₂O, and 2.0 µL of cDNA. Cycling parameters were 95 °C for 30 s and 45 cycles of 95 °C for 5 s, 57 °C

for 20 s, and 72 °C for 30 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference. The primers used were as follows: forward, 5'-TCTGGCCAGCTGTCAGAACTAAAG-3' and reverse, 5'-GTGGGTTGACATTCATGTGAGGA-3' for IP₃RI, and forward, 5'-GACAACTTTGGCATCGTGGA-3' and reverse, 5'-GACAACTTTGGCATCGTGGA-3' for GAPDH. The double-standard curve method was used to determine the relative IP₃RI mRNA expression.

Statistical analysis

All statistical analyses were performed using SPSS 13.0 software. Numerical data, expressed as mean ± standard error of the mean, were compared using analysis of variance. *P* values < 0.05 were considered statistically significant.

RESULTS

Successful induction of HRS in rats with D-GalN/LPS

Following intravenous injection of D-GalN at 400 mg/kg body weight combined with LPS at 32 µg/kg in male SD rats, HRS was successfully induced. Twelve hours after injection, glomerular filtration rate (GFR) significantly decreased, liver and kidney function were severely impaired, and serum biochemical indices, such as alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (Cr), exhibited significant changes. Hematoxylin-eosin staining showed massive hepatocyte necrosis with severe hemorrhage (Figure 1), while renal tissue had a normal morphology at the various time points (Figure 2). These changes were consistent with the clinical features of HRS.

Western blot analysis of IP₃RI protein expression

IP₃RI (230 kDa) and β-actin (45 kDa) were detected in all groups. Densitometry analyses showed that IP₃RI protein expression was significantly elevated in group G/L compared with group NS. This elevation began at 3 h (1.46 ± 0.07 vs 1.00 ± 0.05, *P* = 0.011), became obvious at 9 h, and reached a peak at 12 h (2.89 ± 0.14 vs 1.00 ± 0.05, *P* = 0.000) (Figure 3A).

At 12 h, the expression of IP₃RI protein in the kidney was significantly higher in group G/L than in groups G (1.17 ± 0.08) and L (1.02 ± 0.09) (*P* = 0.000 for both), thus excluding the impact of D-GalN or LPS on the expression of IP₃RI protein. There was no significant difference in IP₃RI protein expression between groups G and L (*P* = 0.245) or between group G or L and group NS (*P* > 0.05 for both) (Figure 3B).

RT-PCR analysis of IP₃RI mRNA expression

IP₃RI mRNA expression was significantly elevated in group G/L compared with group NS. This elevation began at 3 h (2.89 ± 0.51 vs 1.00 ± 0.00, *P* = 0.05), became obvious at 6 h (5.01 ± 0.38, *P* = 0.000), and reached a peak at 9 h (9.96 ± 0.63, *P* = 0.000). IP₃RI mRNA expression began to decline at 12 h, and at 24

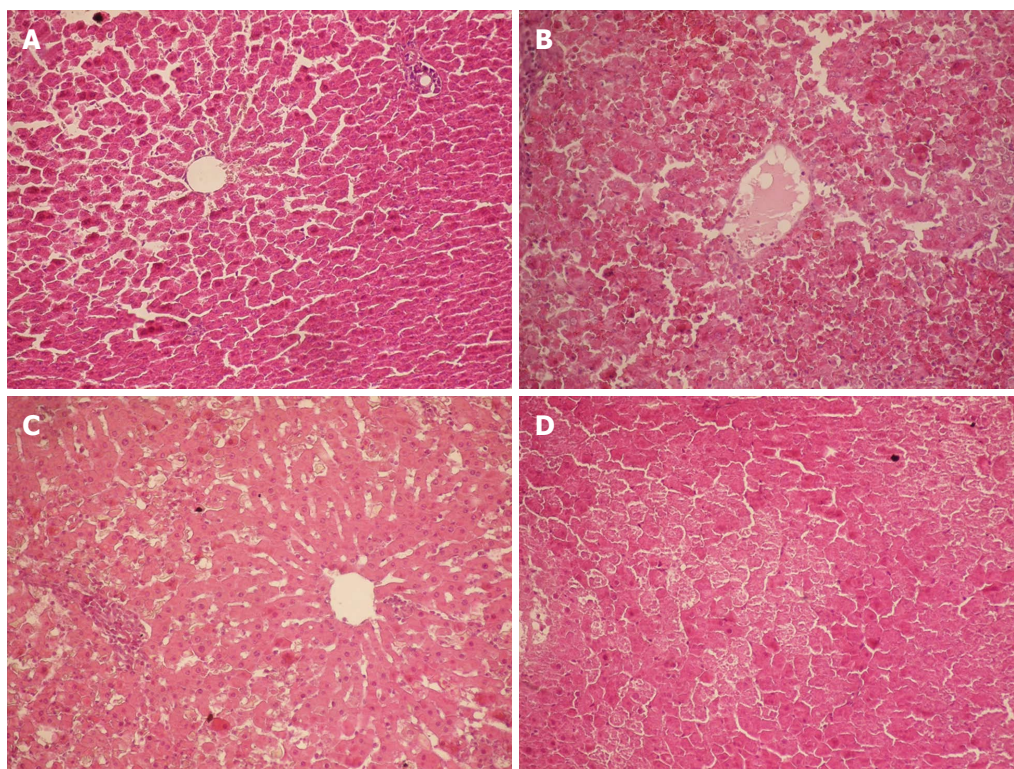


Figure 1 Histopathology of the liver (HE staining, × 200). A: Group Normal Saline (NS). Normal hepatocytes were arranged in cords; B: Group D-galactosamine (D-GalN) plus lipopolysaccharide (LPS) (G/L). At 12 h, massive hepatocyte necrosis with severe hemorrhage developed; C: Group D-GalN (G). At 12 h, spotty hepatocyte necrosis was observed; D: Group LPS (L). At 12 h, hepatocytes began to develop necrosis, with incomplete necrosis visible.

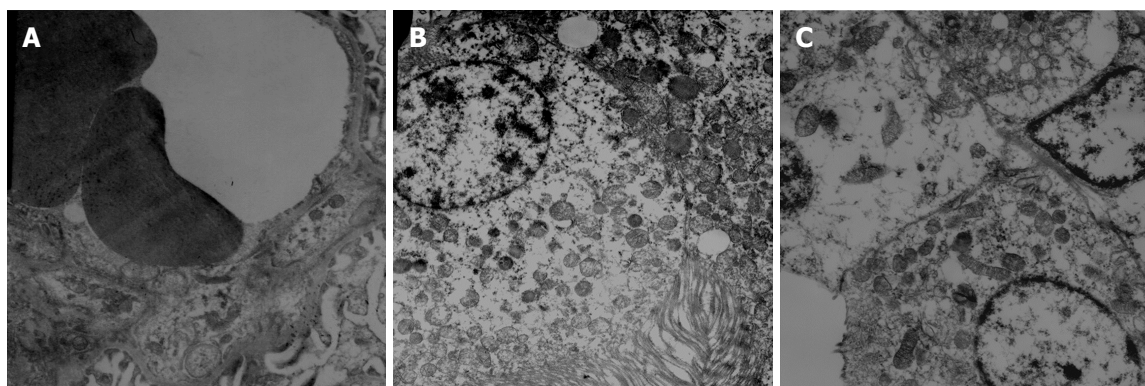


Figure 2 Histopathology of the kidney. A: The glomerular basement membrane of the kidney was intact, and the foot processes of podocytes and fenestra of endothelial cells were clearly visible; B: The basal part of proximal tubule cubical epithelial cells had abundant plasma membrane infolding, which was rich in longitudinally arranged mitochondria with intact cristae. On the free surface of proximal tubule cubical epithelial cells, microvilli were long and dense; C: The basal part of distal tubule cubical epithelial cells also had abundant plasma membrane infolding, which was rich in mitochondria. On the free surface of distal tubule cubical epithelial cells, microvilli were short and sparse.

h, it returned to the level observed at 6 h. IP₃RI mRNA expression at 9 h was significantly higher in group G/L than in groups G (1.43 ± 0.18) and L (1.29 ± 0.17) ($P = 0.000$ for both; Figure 4). IP₃RI mRNA expression did not differ significantly between group G or L and group NS ($P > 0.05$ for both).

DISCUSSION

HRS is one of the most common and severe complications of fulminant liver failure (FHF) and an advanced

liver disease, with approximately 55% of FHF patients developing HRS^[19,20]. The pathogenesis of HRS is still not completely clear, although it is believed to be associated with excessive renal vascular contraction, insufficient renal blood perfusion, sympathetic nervous system activation, and increased synthesis of vasoactive substances, all of which make the kidneys more sensitive to low perfusion^[21,22]. Renal blood flow and GFR decrease significantly in HRS due to renal vasoconstriction, and many factors are involved in this process. A significant increase in vasoconstricting factors

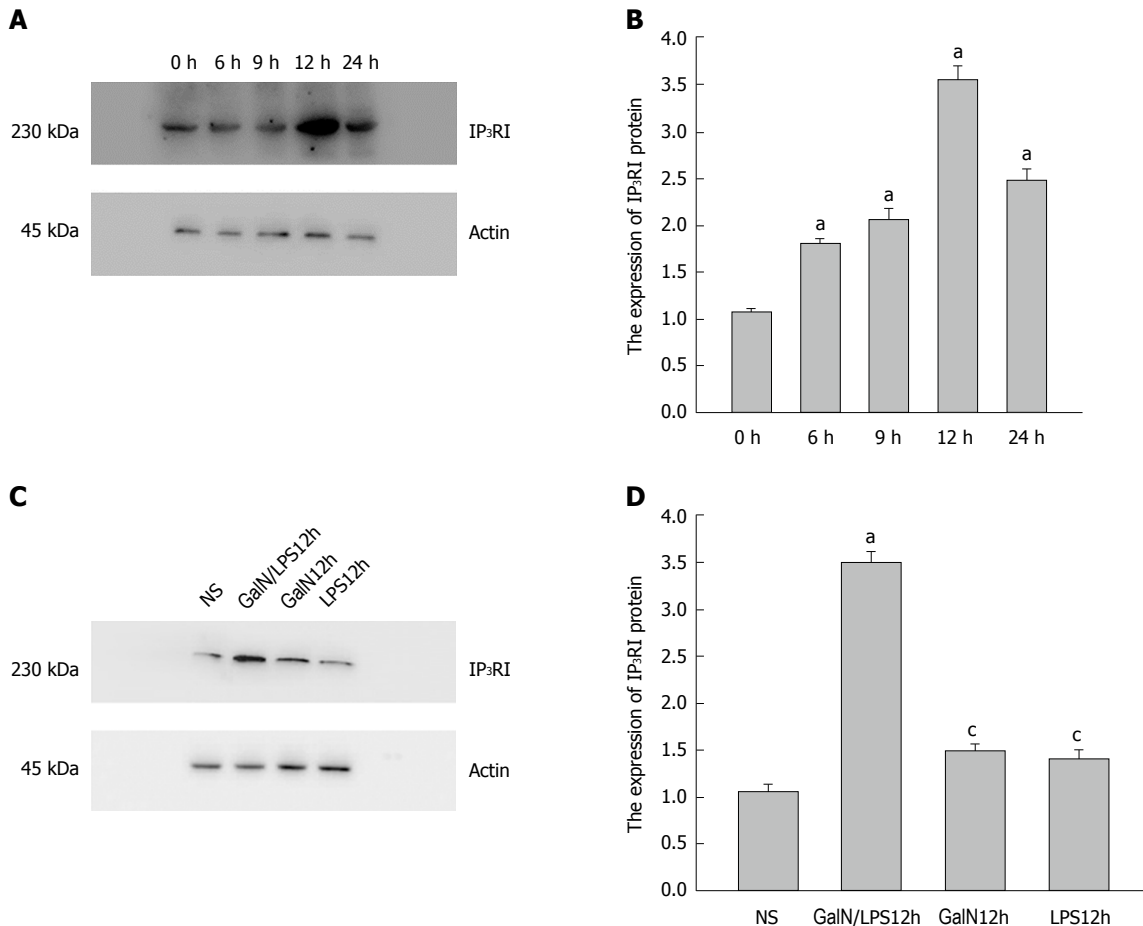


Figure 3 Expression of type I inositol 1,4,5-trisphosphate receptor (IP₃RI) protein in the kidney of rats in each group. A: The expression of IP₃RI protein in the kidney significantly increased in group D-galactosamine (D-GalN) plus lipopolysaccharide (LPS) (G/L), and was especially prominent at 12 h (^a*P* < 0.05 vs group Normal Saline (NS)). B: The expression of IP₃RI protein in the kidney significantly increased in group G/L compared with the other groups (^a*P* < 0.05 vs group NS, ^c*P* < 0.05 vs group G/L).

[e.g., endothelin (ET) and angiotensin II] in the blood not only leads to renal vascular contraction, but also decreases the glomerular filtration coefficient (K_f) and GFR^[23,24]. The contraction of VSMCs results in reduced renal blood flow, while GMC contraction reduces the glomerular filtration fraction and coefficient. As both VSMCs and GMCs are extremely sensitive to vasoactive substances, GFR is significantly decreased in HRS. ET and angiotensin II are important renal vasoconstricting factors, and they activate Ca^{2+} channels *via* the IP₃-IP₃Rs pathway. IP₃Rs is the intracellular calcium reservoir, which is present mainly in the endoplasmic reticulum and on the membrane, directly or indirectly mediating the calcium influx^[25-27]. In addition, IP₃Rs is also present in the nucleus, participating in nuclear calcium release and regulating gene expression^[28,29]. When IP₃ binds to IP₃Rs, a conformational change in IP₃Rs occurs, the calcium channel is open, and the calcium reserve in the endoplasmic reticulum is released into the cytoplasm. As a result, cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) increases, thus causing cell contraction^[30-32]. Therefore, IP₃Rs mediates an important Ca^{2+} signaling pathway in the cell, and the expression of IP₃Rs is closely related

to the sensitivity of the kidney to vasoconstrictors^[33-35]. IP₃Rs has four types of ligand binding sites associated with calcium channels^[36-39], and renal IP₃RI is mainly found in GMCs and VSMCs, and there is almost no IP₃RI on the surface of other renal cells^[40,41]. Therefore, the expression levels of IP₃RI in renal GMCs and VSMCs may be related to renal vasoconstriction. As the opening of IP₃-IP₃Rs channels can increase intracellular $[Ca^{2+}]_i$, theoretically the expression level of IP₃RI is closely related to the intracellular $[Ca^{2+}]_i$ level. Wang *et al* observed increased expression of IP₃RI in the glomerular capillary loops and anterior artery of rats with liver cirrhosis by immunohistochemistry. However, it is unknown whether the expression of IP₃RI increases in FHF. To answer this question, we detected IP₃RI expression in the renal tissue of a rat model of FHF at different time points at both the protein and mRNA levels using Western blot and real-time quantitative PCR, respectively.

Semi-quantitative Western blot analysis demonstrated that IP₃RI protein expression was low in normal kidney tissue. Following treatment with D-GalN plus LPS, IP₃RI protein expression began to rise at 3 h and

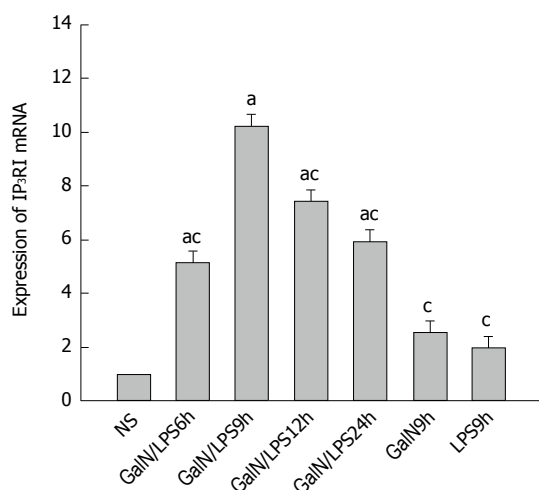


Figure 4 Expression of type 1 inositol 1,4,5-trisphosphate receptor (IP₃RI) mRNA in the kidney of rats in group D-galactosamine (D-GalN) plus lipopolysaccharide (LPS) (G/L). The expression of IP₃RI mRNA in the kidney significantly increased in group G/L, and was especially prominent at 12 h (^a*P* < 0.05 vs group Normal Saline (NS), ^c*P* < 0.05 vs group G/L).

reached a peak at 12 h. Interestingly, liver and kidney dysfunction and hepatocyte necrosis were most severe and blood TNF- α and ET-1 levels were highest at 12 h, which were concomitant with the elevation of IP₃RI protein expression in the kidney^[42,43]. By treating the animals with D-GalN or LPS alone, we excluded the effect of these drugs on IP₃RI protein expression. As HRS developed 12 h after D-GalN/LPS administration, we compared the IP₃RI protein expression at this time point among the groups. The results showed that IP₃RI protein expression was high in group G/L and low in groups G, L, and NS.

In order to understand whether IP₃RI protein expression in the kidney is regulated at the transcriptional level, real-time quantitative PCR was performed. The fluorescent dye SYBR Green I^[44,45] added to the PCR reaction system can be incorporated into double-stranded DNA with PCR amplification and markedly enhance fluorescence^[46-48]. The relative expression levels of these two parameters were calculated by the housekeeping gene GAPDH. It was found that the relative expression of IP₃RI mRNA to GAPDH mRNA began to rise 3 h after D-GalN and LPS administration, but the protein level did not rise at this time point. At 9 h, the expression level of IP₃RI mRNA reached the highest level. Although the protein level was also high at this time point, it was lower than that at 12 h. The expression of IP₃RI mRNA began to decrease, but it was still significantly higher than that in the control group. These changes can be explained from two aspects. On the one hand, IP₃RI mRNA expression may be prior to protein expression, which is associated not only with the translation efficiency and the speed of mRNA degradation, but also with the rate of protein degradation. On the other hand, the protein synthesis process also includes the assembly and translocation of proteins in ribosomes, which may affect the final expression of IP₃RI protein.

In conclusion, joint D-GalN/LPS administration can induce HRS in SD rats at 12 h, which is concomitant with peak IP₃RI protein expression in the kidney. Increased IP₃RI protein expression may be regulated at the transcriptional level. Thus, increased expression of IP₃RI may be closely associated with HRS development and progression.

ARTICLE HIGHLIGHTS

Research background

Hepatorenal syndrome (HRS) is one of the common and severe complications of liver failure and advanced liver disease, with approximately 55% of these patients developing this severe complication. At present, HRS has unclear pathogenesis, limited treatment options, and poor therapeutic efficacy. Once renal dysfunction aggravates rapidly, 60%-80% of patients with HRS will die. Therefore, elucidating the mechanism underlying the development and progression of HRS and taking effective preventive and therapeutic measures may improve the success rate of rescue, the incidence rate, and the mortality rate of HRS.

Research motivation

To detect the protein and mRNA expression of type 1 inositol 1,4,5-trisphosphate receptor (IP₃RI) in the kidney of rats with HRS by Western blot and real-time PCR.

Research objectives

To explore whether high expression of renal IP₃RI is associated with Ca²⁺ influx in vascular smooth muscle cells of glomerular afferent arteries and glomerular mesangial cells in rats with HRS.

Research methods

D-galactosamine (D-GalN) and/or lipopolysaccharide (LPS) were used to treat male Sprague-Dawley (SD) rats via the tail vein. Twelve hours after injection, massive hepatocyte necrosis with severe hemorrhage occurred in the liver, while renal tissue had a normal morphology. In addition, liver and kidney function was impaired severely, and serum biochemical indexes exhibited significant changes. These changes were consistent with the clinical features of HRS. Western blot and real-time PCR were then used to detect the protein and mRNA expression of renal IP₃RI, respectively.

Research results

IP₃RI protein expression was significantly elevated in rats with HRS. The elevation began at 3 h and reached the peak at 12 h. IP₃RI mRNA expression was also significantly elevated in rats with HRS. The elevation began at 3 h and peaked at 9 h.

Research conclusions

Joint D-GalN/LPS administration can induce HRS in SD rats at 12 h, which is concomitant with peaked IP₃RI protein and mRNA expression in the kidney. Increased expression of IP₃RI may be closely associated with HRS development and progression.

Research perspectives

Our results suggest that IP₃RI may be a signal molecule involved in the reduction of renal blood flow induced by renal vasoconstriction in HRS, thus providing a theoretical basis for further research of the pathogenesis of HRS. Gene silencing technology may be adopted to further elucidate the role of IP₃RI in the pathogenesis of HRS.

REFERENCES

- 1 **Hernaez R**, Solà E, Moreau R, Ginès P. Acute-on-chronic liver failure: an update. *Gut* 2017; **66**: 541-553 [PMID: 28053053 DOI: 10.1136/gut-2016-313454]

- 10.1136/gutjnl-2016-312670]
- 2 **de Mattos ÁZ**, de Mattos AA, Méndez-Sánchez N. Hepatorenal syndrome: Current concepts related to diagnosis and management. *Ann Hepatol* 2016; **15**: 474-481 [PMID: 27236146]
- 3 **Bittencourt PL**, Farias AQ, Terra C. Renal failure in cirrhosis: Emerging concepts. *World J Hepatol* 2015; **7**: 2336-2343 [PMID: 26413223 DOI: 10.4254/wjh.v7.i21.2336]
- 4 **Khan MQ**, Anand V, Hessefort N, Hassan A, Ahsan A, Sonnenberg A, Fimmel CJ. Utility of Electronic Medical record-based Fibrosis Scores in Predicting Advanced Cirrhosis in Patients with Hepatitis C Virus Infection. *J Transl Int Med* 2017; **5**: 43-48 [PMID: 28680838 DOI: 10.1515/jtim-2017-0011]
- 5 **Torres-Valadez R**, Roman S, Jose-Abrego A, Sepulveda-Villegas M, Ojeda-Granados C, Rivera-Iñiguez I, Panduro A. Early Detection of Liver Damage in Mexican Patients with Chronic Liver Disease. *J Transl Int Med* 2017; **5**: 49-57 [PMID: 28680839 DOI: 10.1515/jtim-2017-0003]
- 6 **Abenavoli L**, Milic N, Luzzza F, Boccuto L, De Lorenzo A. Polyphenols Treatment in Patients with Nonalcoholic Fatty Liver Disease. *J Transl Int Med* 2017; **5**: 144-147 [PMID: 29164049 DOI: 10.1515/jtim-2017-0027]
- 7 **Elbahrawy A**, Elwassief A, Abdallah AM, Kasem A, Mostafa S, Makboul K, Ali MS, Alashker A, Eliwa AM, Shahbah H, Othman MA, Morsy MH, Abdelbaseer MA, Abdelhafeez H. Hepatitis C Virus Exposure Rate among Health-care Workers in Rural Lower Egypt Governorates. *J Transl Int Med* 2017; **5**: 164-168 [PMID: 29085789 DOI: 10.1515/jtim-2017-0024]
- 8 **Das UN**. Renin-angiotensin-aldosterone system in insulin resistance and metabolic syndrome. *J Transl Int Med* 2016; **4**: 66-72 [PMID: 28191524 DOI: 10.1515/jtim-2016-0022]
- 9 **Bao H**, Peng A. The Green Tea Polyphenol(-)-epigallocatechin-3-gallate and its beneficial roles in chronic kidney disease. *J Transl Int Med* 2016; **4**: 99-103 [PMID: 28191529 DOI: 10.1515/jtim-2016-0031]
- 10 **Wang L**, Mohan C. Contrast-enhanced ultrasound: A promising method for renal microvascular perfusion evaluation. *J Transl Int Med* 2016; **4**: 104-108 [PMID: 28191530 DOI: 10.1515/jtim-2016-0033]
- 11 **Dundar HZ**, Yilmazlar T. Management of hepatorenal syndrome. *World J Nephrol* 2015; **4**: 277-286 [PMID: 25949942 DOI: 10.5527/wjn.v4.i2.277]
- 12 **Colle I**, Laterre PF. Hepatorenal syndrome: the clinical impact of vasoactive therapy. *Expert Rev Gastroenterol Hepatol* 2018; **12**: 173-188 [PMID: 29258378 DOI: 10.1080/17474124.2018.1417034]
- 13 **Geyer M**, Huang F, Sun Y, Vogel SM, Malik AB, Taylor CW, Komarova YA. Microtubule-Associated Protein EB3 Regulates IP₃ Receptor Clustering and Ca(2+) Signaling in Endothelial Cells. *Cell Rep* 2015; **12**: 79-89 [PMID: 26119739 DOI: 10.1016/j.celrep.2015.06.001]
- 14 **Taylor CW**, Tovey SC, Rossi AM, Lopez Sanjurjo CI, Prole DL, Rahman T. Structural organization of signalling to and from IP₃ receptors. *Biochem Soc Trans* 2014; **42**: 63-70 [PMID: 24450629 DOI: 10.1042/BST20130205]
- 15 **Chandrasekhar R**, Alzayady KJ, Yule DI. Using concatenated subunits to investigate the functional consequences of heterotetrameric inositol 1,4,5-trisphosphate receptors. *Biochem Soc Trans* 2015; **43**: 364-370 [PMID: 26009177 DOI: 10.1042/BST20140287]
- 16 **Alzayady KJ**, Wang L, Chandrasekhar R, Wagner LE 2nd, Van Petegem F, Yule DI. Defining the stoichiometry of inositol 1,4,5-trisphosphate binding required to initiate Ca²⁺ release. *Sci Signal* 2016; **9**: ra35 [PMID: 27048566 DOI: 10.1126/scisignal.aad6281]
- 17 **Ambudkar IS**. Ca²⁺ signaling and regulation of fluid secretion in salivary gland acinar cells. *Cell Calcium* 2014; **55**: 297-305 [PMID: 24646566 DOI: 10.1016/j.ceca.2014.02.009]
- 18 **Provence A**, Rovner ES, Petkov GV. Regulation of transient receptor potential melastatin 4 channel by sarcoplasmic reticulum inositol trisphosphate receptors: Role in human detrusor smooth muscle function. *Channels (Austin)* 2017; **11**: 459-466 [PMID: 28644055 DOI: 10.1080/19336950.2017.1341023]
- 19 **Bajaj JS**, O'Leary JG, Reddy KR, Wong F, Biggins SW, Patton H, Fallon MB, Garcia-Tsao G, Maliakkal B, Malik R, Subramanian RM, Thacker LR, Kamath PS; North American Consortium For The Study Of End-Stage Liver Disease (NACSELD). Survival in infection-related acute-on-chronic liver failure is defined by extrahepatic organ failures. *Hepatology* 2014; **60**: 250-256 [PMID: 24677131 DOI: 10.1002/hep.27077]
- 20 **Lenz K**, Buder R, Kapun L, Voglmayr M. Treatment and management of ascites and hepatorenal syndrome: an update. *Therap Adv Gastroenterol* 2015; **8**: 83-100 [PMID: 25729433 DOI: 10.1177/1756283X14564673]
- 21 **Piano S**, Schmidt HH, Ariza X, Amoros A, Romano A, Hüsing-Kabar A, Solà E, Gerbes A, Bernardi M, Alessandria C, Scheiner B, Tonon M, Maschmeier M, Solè C, Trebicka J, Gustot T, Nevens F, Arroyo V, Gines P, Angeli P; EASL CLIF Consortium, European Foundation for the Study of Chronic Liver Failure (EF Clif). Association Between Grade of Acute on Chronic Liver Failure and Response to Terlipressin and Albumin in Patients With Hepatorenal Syndrome. *Clin Gastroenterol Hepatol* 2018; Epub ahead of print [PMID: 29391267 DOI: 10.1016/j.cgh.2018.01.035]
- 22 **Tsien CD**, Rabie R, Wong F. Acute kidney injury in decompensated cirrhosis. *Gut* 2013; **62**: 131-137 [PMID: 22637695 DOI: 10.1136/gutjnl-2011-301255]
- 23 **Bekpinar S**, Vardagli D, Unlucerci Y, Can A, Uysal M, Gurdol F. Effect of rosiglitazone on asymmetric dimethylarginine metabolism in thioacetamide-induced acute liver injury. *Pathophysiology* 2015; **22**: 153-157 [PMID: 26224212 DOI: 10.1016/j.pathophys.2015.06.003]
- 24 **Sridharan K**, Sivaramakrishnan G. Vasoactive Agents for Hepatorenal Syndrome: A Mixed Treatment Comparison Network Meta-Analysis and Trial Sequential Analysis of Randomized Clinical Trials. *J Gen Intern Med* 2018; **33**: 97-102 [PMID: 28924736 DOI: 10.1007/s11606-017-4178-8]
- 25 **Ullah G**, Ullah A. Mode switching of Inositol 1,4,5-trisphosphate receptor channel shapes the spatiotemporal scales of Ca²⁺ signals. *J Biol Phys* 2016; **42**: 507-524 [PMID: 27154029 DOI: 10.1007/s10867-016-9419-2]
- 26 **Alzayady KJ**, Sebé-Pedrós A, Chandrasekhar R, Wang L, Ruiz-Trillo I, Yule DI. Tracing the Evolutionary History of Inositol, 1, 4, 5-Trisphosphate Receptor: Insights from Analyses of Capsaspora owczarzaki Ca²⁺ Release Channel Orthologs. *Mol Biol Evol* 2015; **32**: 2236-2253 [PMID: 25911230 DOI: 10.1093/molbev/msv098]
- 27 **Bánsághi S**, Golenár T, Madesh M, Csordás G, RamachandraRao S, Sharma K, Yule DI, Joseph SK, Hajnóczky G. Isoform- and species-specific control of inositol 1,4,5-trisphosphate (IP₃) receptors by reactive oxygen species. *J Biol Chem* 2014; **289**: 8170-8181 [PMID: 24469450 DOI: 10.1074/jbc.M113.504159]
- 28 **Okeke E**, Parker T, Dingsdale H, Concannon M, Awais M, Voronina S, Molgó J, Begg M, Metcalf D, Knight AE, Sutton R, Haynes L, Tepikin AV. Epithelial-mesenchymal transition, IP₃ receptors and ER-PM junctions: translocation of Ca²⁺ signalling complexes and regulation of migration. *Biochem J* 2016; **473**: 757-767 [PMID: 26759379 DOI: 10.1042/BJ20150364]
- 29 **Subedi KP**, Son MJ, Chidipi B, Kim SW, Wang J, Kim KH, Woo SH, Kim JC. Signaling Pathway for Endothelin-1- and Phenylephrine-Induced cAMP Response Element Binding Protein Activation in Rat Ventricular Myocytes: Role of Inositol 1,4,5-Trisphosphate Receptors and CaMKII. *Cell Physiol Biochem* 2017; **41**: 399-412 [PMID: 28214885 DOI: 10.1159/000456422]
- 30 **Fan G**, Baker ML, Wang Z, Baker MR, Sinyagovskiy PA, Chiu W, Ludtke SJ, Serysheva II. Gating machinery of InsP₃R channels revealed by electron cryomicroscopy. *Nature* 2015; **527**: 336-341 [PMID: 26458101 DOI: 10.1038/nature15249]
- 31 **Seo MD**, Velamakanni S, Ishiyama N, Stathopoulos PB, Rossi AM, Khan SA, Dale P, Li C, Ames JB, Ikura M, Taylor CW. Structural and functional conservation of key domains in InsP₃ and ryanodine receptors. *Nature* 2012; **483**: 108-112 [PMID: 22286060 DOI: 10.1038/nature10751]

- 32 **Alzayady KJ**, Wagner LE 2nd, Chandrasekhar R, Monteagudo A, Godiska R, Tall GG, Joseph SK, Yule DI. Functional inositol 1,4,5-trisphosphate receptors assembled from concatenated homo- and heteromeric subunits. *J Biol Chem* 2013; **288**: 29772-29784 [PMID: 23955339 DOI: 10.1074/jbc.M113.502203]
- 33 **Cao P**, Falcke M, Sneyd J. Mapping Interpuff Interval Distribution to the Properties of Inositol Trisphosphate Receptors. *Biophys J* 2017; **112**: 2138-2146 [PMID: 28538151 DOI: 10.1016/j.bpj.2017.03.019]
- 34 **Chandrasekhar R**, Alzayady KJ, Wagner LE 2nd, Yule DI. Unique Regulatory Properties of Heterotetrameric Inositol 1,4,5-Trisphosphate Receptors Revealed by Studying Concatenated Receptor Constructs. *J Biol Chem* 2016; **291**: 4846-4860 [PMID: 26755721 DOI: 10.1074/jbc.M115.705301]
- 35 **Yang J**, Vais H, Gu W, Foskett JK. Biphasic regulation of InsP₃ receptor gating by dual Ca²⁺ release channel BH3-like domains mediates Bcl-xL control of cell viability. *Proc Natl Acad Sci USA* 2016; **113**: E1953-E1962 [PMID: 26976600 DOI: 10.1073/pnas.1517935113]
- 36 **Sun MY**, Geyer M, Komarova YA. IP₃ receptor signaling and endothelial barrier function. *Cell Mol Life Sci* 2017; **74**: 4189-4207 [PMID: 28803370 DOI: 10.1007/s00018-017-2624-8]
- 37 **Golebiewska U**, Kay JG, Masters T, Grinstein S, Im W, Pastor RW, Scarlata S, McLaughlin S. Evidence for a fence that impedes the diffusion of phosphatidylinositol 4,5-bisphosphate out of the forming phagosomes of macrophages. *Mol Biol Cell* 2011; **22**: 3498-3507 [PMID: 21795401 DOI: 10.1091/mbc.E11-02-0114]
- 38 **Gulyás G**, Tóth JT, Tóth DJ, Kurucz I, Hunyady L, Balla T, Várnai P. Measurement of inositol 1,4,5-trisphosphate in living cells using an improved set of resonance energy transfer-based biosensors. *PLoS One* 2015; **10**: e0125601 [PMID: 25932648 DOI: 10.1371/journal.pone.0125601]
- 39 **Takeda Y**, Shimayoshi T, Holz GG, Noma A. Modeling analysis of inositol 1,4,5-trisphosphate receptor-mediated Ca²⁺ mobilization under the control of glucagon-like peptide-1 in mouse pancreatic β -cells. *Am J Physiol Cell Physiol* 2016; **310**: C337-C347 [PMID: 26741144 DOI: 10.1152/ajpcell.00234.2015]
- 40 **Bojjireddy N**, Botyanszki J, Hammond G, Creech D, Peterson R, Kemp DC, Snead M, Brown R, Morrison A, Wilson S, Harrison S, Moore C, Balla T. Pharmacological and genetic targeting of the PI4KA enzyme reveals its important role in maintaining plasma membrane phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate levels. *J Biol Chem* 2014; **289**: 6120-6132 [PMID: 24415756 DOI: 10.1074/jbc.M113.531426]
- 41 **Wagner LE 2nd**, Yule DI. Differential regulation of the InsP₃ receptor type-1 and -2 single channel properties by InsP₃, Ca²⁺ and ATP. *J Physiol* 2012; **590**: 3245-3259 [PMID: 22547632 DOI: 10.1113/jphysiol.2012.228320]
- 42 **Wang JB**, Wang DL, Wang HT, Wang ZH, Wen Y, Sun CM, Zhao YT, Wu J, Liu P. Tumor necrosis factor- α -induced reduction of glomerular filtration rate in rats with fulminant hepatic failure. *Lab Invest* 2014; **94**: 740-751 [PMID: 24887412 DOI: 10.1038/labinvest.2014.71]
- 43 **Wang JB**, Wang HT, Li LP, Yan YC, Wang W, Liu JY, Zhao YT, Gao WS, Zhang MX. Development of a rat model of D-galactosamine/lipopolysaccharide induced hepatorenal syndrome. *World J Gastroenterol* 2015; **21**: 9927-9935 [PMID: 26379397 DOI: 10.3748/wjg.v21.i34.9927]
- 44 **Barbau-Piednoir E**, Bertrand S, Mahillon J, Roosens NH, Botteldoorn N. SYBR®Green qPCR Salmonella detection system allowing discrimination at the genus, species and subspecies levels. *Appl Microbiol Biotechnol* 2013; **97**: 9811-9824 [PMID: 24113820 DOI: 10.1007/s00253-013-5234-x]
- 45 **Barbau-Piednoir E**, Denayer S, Botteldoorn N, Dierick K, De Keersmaecker SCJ, Roosens NH. Detection and discrimination of five E. coli pathotypes using a combinatory SYBR® Green qPCR screening system. *Appl Microbiol Biotechnol* 2018; **102**: 3267-3285 [PMID: 29460001 DOI: 10.1007/s00253-018-8820-0]
- 46 **Barbau-Piednoir E**, Botteldoorn N, Mahillon J, Dierick K, Roosens NH. Fast and discriminative CoSYPS detection system of viable Salmonella spp. and Listeria spp. in carcass swab samples. *Int J Food Microbiol* 2015; **192**: 103-110 [PMID: 25440553 DOI: 10.1016/j.ijfoodmicro.2014.09.018]
- 47 **Acevedo AM**, Perera CL, Vega A, Ríos L, Coronado L, Relova D, Frías MT, Ganges L, Núñez JI, Pérez LJ. A duplex SYBR Green I-based real-time RT-PCR assay for the simultaneous detection and differentiation of Massachusetts and non-Massachusetts serotypes of infectious bronchitis virus. *Mol Cell Probes* 2013; **27**: 184-192 [PMID: 23810983 DOI: 10.1016/j.mcp.2013.06.001]
- 48 **Fellahi S**, El Harrak M, Kuhn JH, Sebbar G, Bouaiti el A, Khataby K, Fihri OF, El Houadfi M, Ennaji MM. Comparison of SYBR green I real-time RT-PCR with conventional agarose gel-based RT-PCR for the diagnosis of infectious bronchitis virus infection in chickens in Morocco. *BMC Res Notes* 2016; **9**: 231 [PMID: 27106608 DOI: 10.1186/s13104-016-2037-z]

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Retrospective Study

Prognostic significance of the fibrinogen-to-albumin ratio in gallbladder cancer patients

Wei-Yu Xu, Hao-Hai Zhang, Jian-Ping Xiong, Xiao-Bo Yang, Yi Bai, Jian-Zhen Lin, Jun-Yu Long, Yong-Chang Zheng, Hai-Tao Zhao, Xin-Ting Sang

Wei-Yu Xu, Hao-Hai Zhang, Jian-Ping Xiong, Xiao-Bo Yang, Yi Bai, Jian-Zhen Lin, Jun-Yu Long, Yong-Chang Zheng, Hai-Tao Zhao, Xin-Ting Sang, Department of Liver Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China

ORCID number: Wei-Yu Xu (0000-0002-2101-4829); Hao-Hai Zhang (0000-0002-5292-6505); Jian-Ping Xiong (0000-0002-6163-2621); Xiao-Bo Yang (0000-0003-1929-8866); Yi Bai (0000-0002-1179-3734); Jian-Zhen Lin (0000-0002-4767-8834); Jun-Yu Long (0000-0001-5745-7165); Yong-Chang Zheng (0000-0002-6956-1186); Hai-Tao Zhao (0000-0002-3444-8044); Xin-Ting Sang (0000-0003-1952-0527).

Author contributions: Xu WY conceived the research, collected the clinical data and wrote the manuscript that led to the submission; Xu WY, Zhang HH, and Xiong JP helped to collect the clinical data and followed up with the patients; Lin JZ, Long JY, and Yang XB helped to analyze the data, Bai Y and Zheng YC revised the manuscript, Zhao HT and Sang XT provided financial support for this work; Zhao HT and Sang XT are co-corresponding authors, and they contributed equally to this work; all authors read and approved the final manuscript.

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Correspondence to: Xin-Ting Sang, MD, Professor, Department of Liver Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 1 Shuaifuyuan, Wangfujing, Beijing 100730, China. sangxt@pumch.cn.

Telephone: +86-10-69156042

Fax: +86-10-69156042

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Abstract

AIM

To investigate the prognostic role of fibrinogen-to-albumin ratio (FAR) on patients with gallbladder cancer (GBC) in this study.

METHODS

One hundred and fifty-four GBC patients were retro-

spectively analyzed, who received potentially curative cholecystectomy in our institute from March 2005 to December 2017. Receiver operating characteristic curve (ROC curve) was used to determine the optimal cut-offs for these biomarkers. In addition, Kaplan-Meier survival analysis as well as multivariate analysis were applied for prognostic analyses.

RESULTS

ROC curve revealed that the optimal cut-off value for FAR was 0.08. FAR was significantly correlated with age ($P = 0.045$), jaundice ($P < 0.001$), differentiation ($P = 0.002$), resection margin status ($P < 0.001$), T stage ($P < 0.001$), TNM stage ($P < 0.001$), and CA199 ($P < 0.001$) as well as albumin levels ($P < 0.001$). Multivariate analysis indicated that the resection margin status [hazard ratio (HR): 2.343, 95% confidence interval (CI): 1.532-3.581, $P < 0.001$], TNM stage ($P = 0.035$), albumin level (HR = 0.595, 95%CI: 0.385-0.921, $P = 0.020$) and FAR (HR: 2.813, 95%CI: 1.765-4.484, $P < 0.001$) were independent prognostic factors in GBC patients.

CONCLUSION

An elevated preoperative FAR was significantly correlated with unfavorable overall survival in GBC patients, while an elevated preoperative albumin level was a protective prognostic factor for patients with GBC. The preoperative FAR could be used to predict the prognosis of GBC patients, which was easily accessible, cost-effective and noninvasive.

Key words: Gallbladder cancer; Fibrinogen; Albumin; Fibrinogen-to-albumin ratio; Prognosis; Survival

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Core tip: The vital prognostic significance of fibrinogen and serum albumin has been confirmed in diverse malignancies, presenting host hemostasis and nutrition, respectively. Moreover, elevated plasma fibrinogen and reduced serum albumin levels are significantly related to shortened survival of cancer patients. It is reported that fibrinogen-to-albumin ratio (FAR) is more potent in predicting cancer patient prognosis than elevated fibrinogen or reduced serum albumin level alone. Nevertheless, there has been no study on the prognostic role of FAR in gallbladder cancer (GBC). Herein, we defined an elevated preoperative FAR, featured by noninvasiveness, cost-effectiveness and easily-accessible, which was a potential prognostic indicator for GBC.

Xu WY, Zhang HH, Xiong JP, Yang XB, Bai Y, Lin JZ, Long JY, Zheng YC, Zhao HT, Sang XT. Prognostic significance of the fibrinogen-to-albumin ratio in gallbladder cancer patients. *World J Gastroenterol* 2018; 24(29): 3281-3292 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i29/3281.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i29.3281>

INTRODUCTION

Gallbladder cancer (GBC) is an uncommon malignancy among all types of cancer, but is the fifth most common gastrointestinal malignancy. Meanwhile, GBC is the most prevalent and aggressive cancer of the biliary tract^[1-3]. Despite recent encouraging progress in the diagnosis and treatment of GBC, it is still a highly lethal disease, with overall 5-year survival rate under 5%^[4]. Only surgical intervention renders probability of a long-term survival, however, most GBC patients generally present at late stage, with unresectable lesion. To be specific, fewer than 20% of cases are amenable to surgical treatment^[5,6].

At present, a few clinicopathological parameters, such as clinical stage, performance status (PS), and pathological classification, have been demonstrated as independent survival predictors in patients harboring various types of common solid tumors^[7]. Nevertheless, despite the wide application of high-resolution imaging systems, it is rather difficult to obtain accurate classification of clinical stage, and objective judgement of PS^[8-10]. In addition, the pathological stage of tumor samples in these subjects is not as informative as that in untreated subjects^[11]. In order to guarantee potent intense neoadjuvant therapy as well as regular follow-up in high-risk subjects, it is necessary to explore a simple and cost-effective predictor for the postoperative overall survival (OS) prior to surgery.

Accumulating evidence has demonstrated that nutritional deficiencies, hemostatic factors and systemic inflammatory response (SIR) are likely to be critically involved in the progression of human malignancies^[12]. Fibrinogen plays an important regulatory role in both inflammation and cancer progression, including proliferation, angiogenesis as well as migration of tumor cells^[13]. Serum albumin levels reflect the SIR of host and nutritional status^[14-16]. Recently, accumulating researches have shown that both fibrinogen and serum albumin are important prognostic predictors in various cancers, and elevated plasma fibrinogen and lower serum albumin levels are significantly correlated with shorter survival in tumor patients^[17-21].

From the results of the above studies, we can naturally hypothesize that the fibrinogen-to-albumin ratio (FAR) might be more powerful than elevated fibrinogen or lower serum albumin level in predicting the prognosis of patients with malignant tumors. In fact, Tan *et al.*^[22] have indicated that the preoperative FAR is an independent prognostic indicator for esophageal squamous-cell carcinoma (ESCC) patients, while Hwang *et al.*^[23] have indicated that the FAR is a more significant prognostic indicator than either indicator alone (elevated fibrinogen or lower serum albumin).

To our knowledge, there are no relevant studies concerning the prognostic significance of FAR in GBC patients. Herein, the study was designed to explore the prognostic roles of the preoperative FAR in GBC in terms of OS.

MATERIALS AND METHODS

Patients

Eligible patients were included in this study according to the following criteria: (1) patients with histological diagnosis of GBC; (2) GBC patients without other coexisting malignancies; (3) patients not undergoing other treatments before enrollment; (4) patients with complete clinical information and available follow-up data; and (5) patients aged > 18 years. The exclusion criteria were listed as follows: (1) patients with acute infection or chronic active inflammatory disease; (2) patients with collagen diseases, anemia and other diseases concerning the hematological system; (3) patients who received anticoagulant treatment or albumin transfusions before treatment; (4) patients with liver disease; and (5) patients with perioperative surgery-associated mortality. As a result, 154 GBC patients were retrospectively included and analyzed, who underwent potential curative resection at Peking Union Medical College Hospital of Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS & PUMC) from January 2005 to May 2017.

Data collection

Baseline clinicopathological characteristics, including age, gender, comorbidities, ABO blood group, pathological classifications, tumor differentiation, resection margin status, maximal tumor diameter, TNM stage, and preoperative CA199, fibrinogen, and albumin levels. Patient age referred to the age at diagnosis of primary GBC. The eighth edition of the American Joint Committee on Cancer (AJCC-8th) TNM classification was utilized for TNM stage.

Ethical statement

The study was approved by the Medical Ethics Committee of Peking Union Medical College Hospital of CAMS & PUMC. All patients signed written informed consent. The study was carried out according to the ethical standard of the World Medical Association Declaration of Helsinki^[24].

Fibrinogen and albumin measurements

Blood specimens were collected before breakfast within seven days before surgery, in order to assess the preoperative plasma fibrinogen and serum albumin concentrations. Afterwards, Datafai Fibrinogen (Sysmex Corporation, Kobe, Japan) and CA7000 analyzer (Sysmex Corporation, Kobe, Japan) were employed to assess fibrinogen level using the previously-mentioned Clauss method^[25]. The normal reference values of plasma fibrinogen and serum albumin were 2–4 g/L and 35–51 g/L, respectively, according to relevant instructions.

FAR

FAR was defined by dividing the preoperative fibrinogen level by the preoperative serum albumin level.

Treatments and Follow up

All subjects received potential curative gallbladder resection at Peking Union Medical College Hospital of CAMS & PUMC. The extent of resection was classified as modified radical cholecystectomy or radical cholecystectomy and systemic therapy according to the extent of tumor invasion, which was identified by preoperative auxiliary examination results. Follow-up visits in our center were carried out every three months for the first two years, every six months for the third year and annually thereafter. The follow-up period was defined from the date of surgery to death or the last follow-up visit.

Statistical analysis

The continuous data with normal distribution were shown as the mean \pm standard deviation (Kolmogorov-Smirnov test, $P > 0.05$), and those with abnormal distribution were expressed as the median (minimum–maximum). Frequencies and percentages were used for the categorical variables. Chi-square test or Fisher's exact test was utilized to assess differences in baseline clinicopathological characteristics between groups. OS referred to the duration from the date of surgery to death or the last follow-up visit. The optimal cut-off values of fibrinogen, albumin and FAR were identified by the receiver operating characteristic (ROC) curve. Kaplan-Meier method was used to generate the survival curves, followed by analysis by log-rank test. Additionally, multivariate Cox proportional hazards model was used to further assess those significant factors indicated by univariate analysis. SPSS version 24.0 (IBM Corp., Armonk, NY, United States) was utilized for statistical analysis. A two-sided $P < 0.05$ was considered as statistical significance, and 95% confidence intervals (CIs) were calculated.

RESULTS

Patient characteristics

All the 154 GBC patients in this study were treated at Peking Union Medical Hospital from January 2005 to May 2017. The median follow-up period was 17 mo. In total, 103 subjects died during the follow-up period, with an estimated median OS of 14.5 mo (range: 0.5–153.0 mo). The 1- and 2-year survival rates were 55.8% and 35.7%, respectively. The clinical data of all patients who met all criteria were analyzed. Among these patients, the median age at diagnosis was 64 years old (range: 29–85 years old), of whom, 98 (63.6%) were > 60 years old. Ninety-one (59.1%) patients were female. One hundred fifty (97.4%) patients were pathologically diagnosed with adenocarcinoma, three (1.9%) with adenosquamous cell carcinoma and one (0.6%) with papillary carcinoma. Ninety-four (61.0%) patients were histologically diagnosed with moderately or well-differentiated disease. Fifty-eight (37.7%) patients harbored a positive resection margin. According to the

Table 1 Baseline characteristics of 154 gallbladder cancer patients who underwent potential curative cholecystectomy *n* (%)

Characteristic	Patients (<i>n</i> = 154)
Age (yr)	64 (29-85)
≤ 60	56 (36.4)
> 60	98 (63.6)
Sex	
Male	63 (40.9)
Female	91 (59.1)
Cholecystolithiasis	
Absent	79 (51.3)
Present	75 (48.7)
Diabetes	
Absent	116 (75.3)
Present	38 (24.7)
Jaundice	
Absent	129 (83.8)
Present	25 (8.9)
Blood groups	
A	43 (27.9)
B	56 (36.4)
AB	9 (5.8)
O	46 (29.9)
Pathological types	
Adenosquamous carcinoma	3 (1.9)
Adenocarcinoma	150 (97.4)
Papilocarcinoma	1 (0.6)
Degree of differentiation	
Poor	60 (39.0)
Moderate-well	94 (61.0)
Resection margin status	
Negative	96 (62.3)
Positive	58 (37.7)
Maximum tumor diameter (cm)	3 (0.2-13)
≤ 2.45	68 (44.2)
> 2.45	86 (55.8)
T stage	
Tis-T1a	10 (6.5)
T1b-T2b	29 (18.8)
T3	103 (66.9)
T4	12 (7.8)
N stage	
0	98 (63.6)
1	47 (30.5)
2	9 (5.8)
Distant metastasis	
Absent	142 (92.2)
Present	12 (7.8)
TNM stage	
0- I stage	16 (10.4)
II A- II B stage	16 (10.4)
III A- III B stage	92 (59.7)
IV A-IV B stage	30 (19.5)
CA199 (U/mL)	69.3 (0.6-10524)
≤ 39	66 (42.9)
> 39	88 (57.1)
Fibrinogen concentration (g/L)	3.54 (1.71-7.47)
≤ 3.47	75 (48.7)
> 3.47	79 (51.3)
Albumin levels (g/L)	41.0 (20.0-50.0)
≤ 40.5	78 (50.6)
> 40.5	76 (49.4)
FAR	0.09 (0.04-0.25)
≤ 0.08	71 (46.1)
> 0.08	83 (53.9)

FAR: Fibrinogen to albumin ratio.

TNM staging, most patients (59.7%) were classified as stage IIIA-III B. The detailed information of baseline characteristics of patients was shown in Table 1.

The optimal cut-off value of the preoperative fibrinogen concentration, albumin level and FAR for survival analysis

The ROC curves of OS were generated to validate the optimal cut-off values for the preoperative fibrinogen concentration, albumin level and FAR (Figure 1). The median plasma fibrinogen concentration in all patients was 3.54 g/L (range: 1.71-7.47 g/L) (Table 1). As shown in Figure 1A, the area under the curve (AUC) was recorded as 0.735 (95%CI: 0.654-0.816), and the optimal cut-off value of preoperative fibrinogen concentration for OS was 3.47 g/L, with the highest sensitivity and specificity of 0.709 and 0.721, respectively. Based on this cut-off, there were 75 patients (48.7%) with a fibrinogen concentration ≤ 3.47 g/L, and 79 patients (51.3%) with a fibrinogen concentration > 3.47 g/L (Table 2).

The median serum albumin level in all patients was 41.0 g/L (range: 20.0-40.0 g/L) (Table 1). As shown in Figure 1B, the AUC was recorded as 0.648 (95%CI: 0.562-0.735), and the optimal cut-off value of the preoperative albumin level for OS was 40.5 g/L, with the highest sensitivity and specificity of 0.647 and 0.605, respectively. Based on this value, 76 patients (49.4%) had an albumin level ≤ 40.5 g/L, and 78 patients (50.6%) had an albumin level > 40.5 g/L (Table 3).

The median FAR in all patients was 0.09 (range: 0.04-0.25) (Table 1). As shown in Figure 1C, the AUC was recorded as 0.783 (95%CI: 0.707-0.859), and the optimal cut-off value of the preoperative FAR for OS was 0.08, with the highest sensitivity and specificity of 0.779 and 0.765, respectively. Based on this value, 71 patients (46.1%) harbored a FAR value ≤ 0.08, and 83 patients (53.9%) had a FAR value > 0.08 (Table 4).

Correlations of the preoperative fibrinogen concentration, albumin level and FAR with clinicopathological factors

As shown in Table 2, based on the optimal cut-off value for the preoperative fibrinogen concentration, all patients could be divided into the low-value group (≤ 3.47 g/L) or the high-value group (> 3.47 g/L). Higher preoperative fibrinogen concentration was significantly correlated with jaundice ($P = 0.003$), degree of differentiation ($P = 0.048$), resection margin ($P = 0.003$), T stage ($P < 0.001$), TNM stage ($P = 0.011$), CA199 level ($P = 0.005$) as well as FAR ($P < 0.001$). However, there were no significant associations of the preoperative fibrinogen concentration with age, gender, cholecystolithiasis, diabetes, ABO blood group, pathological type, tumor size, N stage, distant metastasis or albumin level ($P > 0.05$). The survival curve stratified by the fibrinogen concentration indicated that GBC subjects with a fibrinogen concentration > 3.47 g/L had shorter OS than those with a fibrinogen concentration ≤ 3.47 g/L (Figure 2A).

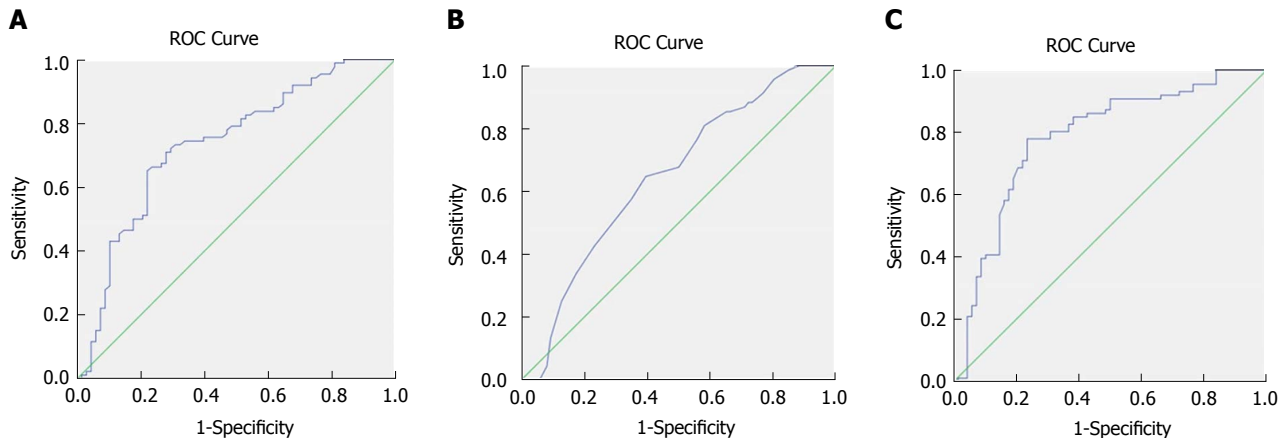


Figure 1 Receiver operating characteristics curve analysis based on fibrinogen (A), albumin (B), and fibrinogen to albumin ratio (C) for overall survival. A: The area under the ROC curve (AUC) indicates the diagnostic power of preoperative plasma fibrinogen concentration. In this model, the optimum cut-off point for fibrinogen concentration was 3.47 g/L, AUC was 0.735 (95%CI: 0.654-0.816), with a sensitivity of 0.709 and a specificity of 0.721 by the Youden index; B: The AUC indicates the diagnostic power of preoperative plasma albumin level. In this model, the optimum cut-off point for albumin level was 40.5 g/L, AUC was 0.648 (95%CI: 0.562-0.735), with a sensitivity of 0.647 and a specificity of 0.605 by the Youden index; C: The AUC indicates the diagnostic power of preoperative FAR. In this model, the optimum cut-off point for FAR was 0.08, AUC was 0.783 (95%CI: 0.707-0.859), with a sensitivity of 0.779 and a specificity of 0.765 by the Youden index. ROC: Receiver operating characteristics curve.

As shown in Table 3, based on the optimal cut-off value for the preoperative albumin level, all patients could be categorized into the low-value group (≤ 40.5 g/L) or high-value group (> 40.5 g/L). Higher preoperative albumin levels were significantly associated with jaundice ($P < 0.001$), ABO blood group ($P = 0.046$), degree of differentiation ($P = 0.047$), resection margin status ($P = 0.008$), T stage ($P = 0.021$), TNM stage ($P = 0.007$), CA199 levels ($P = 0.006$) as well as FAR ($P < 0.001$). The survival curve stratified by the albumin level showed that GBC patients with an albumin level > 40.5 g/L had longer OS than those with an albumin level ≤ 40.5 g/L (Figure 2B).

As shown in Table 4, based on the optimal cut-off value for the preoperative FAR, all patients could be grouped into the low-value group (≤ 0.08) or high-value group (> 0.08). A higher preoperative FAR was significantly correlated with age ($P = 0.045$), jaundice ($P < 0.001$), degree of differentiation ($P = 0.002$), resection margin status ($P < 0.001$), T stage ($P < 0.001$), TNM stage ($P < 0.001$), CA199 level ($P < 0.001$) as well as albumin level ($P < 0.001$). The survival curve stratified by the FAR showed that GBC patients with a FAR > 0.08 harbored worse OS compared to those with a FAR ≤ 0.08 (Figure 2C).

Univariate and multivariate analysis results

Univariate and multivariate analyses for OS prediction in GBC patients are shown in Tables 5 and 6. In the univariate Cox analysis, jaundice (HR: 2.598, 95%CI: 1.644-4.106, $P < 0.001$), degree of differentiation (HR: 1.527, 95%CI: 1.031-2.261, $P = 0.035$), resection margin status (HR: 3.683, 95%CI: 2.468-5.496, $P < 0.001$), T stage ($P < 0.001$), N stage ($P < 0.001$), distant metastasis (HR: 2.550, 95%CI: 1.388-4.684, $P = 0.003$), TNM stage ($P < 0.001$), CA199 level (HR: 3.125, 95%CI: 2.010-4.858, $P < 0.001$), fibrinogen

concentration (HR: 2.795, 95%CI: 1.853-4.214, $P < 0.001$), albumin level (HR: 0.391, 95%CI: 0.259-0.590, $P < 0.001$) and FAR (HR: 4.626, 95%CI: 2.987-7.165, $P < 0.001$) were significant prognostic factors for OS in GBC patients (Table 5), whereas age, gender, cholecystolithiasis, diabetes, ABO blood group, pathological type and maximal tumor diameter were not significant predictors of OS ($P > 0.05$; Table 5). In the multivariate Cox regression analysis, resection margin status (HR: 2.343, 95%CI: 1.532-3.581, $P < 0.001$), TNM stage ($P = 0.035$), and FAR (HR: 2.813, 95%CI: 1.765-4.484, $P < 0.001$) were revealed as independent risk factors for poor OS in GBC patients, and the albumin level (HR: 0.595, 95%CI: 0.385-0.921, $P = 0.020$) was correlated with favorable OS in patients with GBC (Table 6).

Although the multivariate analysis showed that both FAR and albumin level were independent risk factors for the prognosis of GBC patients (Table 6), the AUC of FAR (0.783) was greater than that (0.648) of the albumin level (Figure 1B and C), indicating that the prognostic value of FAR was more powerful than that of the albumin level.

DISCUSSION

In this study, we demonstrate FAR is a significantly independent prognostic indicator for GBC. To our knowledge, it is the first research concerning the prognostic significance of FAR in patients with GBC. Although both FAR and serum albumin level were revealed to be significant prognostic indicators, the AUC of FAR was greater than that of the serum albumin level, and the P value of FAR was smaller than that of the serum albumin level.

In our study, a greater FAR was found to be correlated with a series of important clinicopathological indicators

Table 2 Correlation between fibrinogen concentration and clinicopathological characteristics in gallbladder cancer patients *n* (%)

Characteristics	Fibrinogen concentration		<i>P</i> value
	≤ 3.47 g/L (<i>n</i> = 75)	> 3.47 g/L (<i>n</i> = 79)	
Age (yr)			
≤ 60	31 (20.1)	25 (16.2)	0.243
> 60	44 (28.6)	54 (35.1)	
Sex			
Male	33 (21.4)	30 (19.5)	0.513
Female	42 (27.3)	49 (31.8)	
Cholecystolithiasis			
Absent	38 (24.7)	41 (26.6)	0.878
Present	37 (24.0)	38 (24.7)	
Diabetes			
Absent	57 (37.0)	59 (38.3)	0.850
Present	18 (11.7)	20 (13.0)	
Jaundice			
Absent	68 (44.2)	61 (39.6)	0.029
Present	7 (4.5)	18 (11.7)	
Blood groups			
A	19 (12.3)	24 (15.6)	0.145
B	33 (21.4)	23 (14.9)	
AB	2 (1.3)	7 (4.5)	
O	21 (13.6)	25 (16.2)	
Pathological types			
Adenosquamous carcinoma	0 (0)	3 (1.9)	0.142
Adenocarcinoma	75 (48.7)	75 (48.7)	
Papilocarcinoma	0 (0)	1 (0.6)	
Degree of differentiation			
Poor	23 (14.9)	37 (24.0)	0.048
Moderate-well	52 (33.8)	42 (27.3)	
Resection margin status			
Negative	56 (36.4)	40 (26.4)	0.003
Positive	19 (12.3)	39 (25.3)	
Maximum tumor diameter (cm)			
≤ 2.45	34 (22.1)	34 (22.1)	0.871
> 2.45	41 (26.6)	45 (29.2)	
T stage			
Tis-T1a	8 (5.2)	2 (1.3)	< 0.001
T1b-T2b	22 (14.3)	7 (4.5)	
T3	43 (27.9)	60 (39.0)	
T4	2 (1.3)	10 (6.5)	
N stage			
N0	50 (32.5)	48 (31.2)	0.748
N1	21 (13.6)	26 (16.9)	
N2	4 (2.6)	5 (3.2)	
Distant metastasis			
Absent	69 (44.8)	73 (47.4)	0.925
Present	6 (3.9)	6 (3.9)	
TNM stage			
0- I stage	12 (7.8)	4 (2.6)	0.011
II A- II B stage	12 (7.8)	4 (2.6)	
III A- III B stage	39 (25.3)	53 (34.4)	
IVA-IVB stage	12 (7.8)	18 (11.7)	
CA199 (U/mL)			
≤ 39	41 (26.6)	25 (16.2)	0.005
> 39	34 (22.1)	54 (35.1)	
Albumin levels (g/L)			
≤ 40.5	32 (20.8)	44 (28.6)	0.111
> 40.5	43 (27.9)	35 (22.7)	
FAR			
≤ 0.08	59 (38.3)	12 (7.8)	< 0.001
> 0.08	16 (10.4)	67 (43.5)	

FAR: Fibrinogen to albumin ratio.

Table 3 Correlation between albumin levels and clinicopathological characteristics in gallbladder cancer patients *n* (%)

Characteristics	Albumin levels		<i>P</i> value
	≤ 40.5g/L (<i>n</i> = 76)	> 40.5 g/L (<i>n</i> = 78)	
Age (yr)			
≤ 60	22 (14.3)	34 (22.1)	0.067
> 60	54 (35.1)	44 (28.6)	
Sex			
Male	28 (18.2)	35 (22.7)	0.330
Female	48 (31.2)	43 (27.9)	
Cholecystolithiasis			
Absent	34 (22.1)	45 (29.2)	0.147
Present	42 (27.3)	33 (21.4)	
Diabetes			
Absent	53 (34.4)	63 (40.9)	0.136
Present	23 (14.9)	15 (9.7)	
Jaundice			
Absent	54 (35.1)	75 (48.7)	< 0.001
Present	22 (14.3)	3 (1.9)	
Blood groups			
A	20 (13.0)	23 (14.9)	0.046
B	34 (22.1)	22 (14.3)	
AB	6 (7.9)	3 (3.8)	
O	16 (21.1)	30 (19.5)	
Pathological types			
Adenosquamous carcinoma	0 (0)	3 (1.9)	0.137
Adenocarcinoma	75 (48.7)	75 (48.7)	
Papilocarcinoma	1 (0.6)	0 (0.0)	
Degree of differentiation			
Poor	36 (23.4)	24 (15.6)	0.047
Moderate-well	40 (26.0)	54 (35.1)	
Resection margin status			
Negative	39 (25.3)	57 (37.0)	0.008
Positive	37 (24.0)	21 (13.6)	
Maximum tumor diameter (cm)			
≤ 2.45	36 (23.4)	32 (20.8)	0.516
> 2.45	40 (26.0)	46 (29.9)	
T stage			
Tis-T1a	2 (1.3)	8 (5.2)	0.021
T1b-T2b	9 (5.8)	20 (13.0)	
T3	58 (37.7)	45 (29.2)	
T4	7 (4.5)	5 (3.2)	
N stage			
N0	45 (29.2)	53 (34.4)	0.403
N1	25 (16.2)	22 (14.3)	
N2	6 (3.9)	3 (1.9)	
Distant metastasis			
Absent	67 (43.5)	75 (48.7)	0.077
Present	9 (5.8)	3 (1.9)	
TNM stage			
0- I stage	3 (1.9)	13 (8.4)	0.007
II A- II B stage	6 (3.9)	10 (6.5)	
III A- III B stage	46 (29.9)	46 (29.9)	
IVA-IVB stage	21 (13.6)	9 (5.8)	
CA199 (U/mL)			
≤ 39	24 (15.6)	42 (27.3)	0.006
> 39	52 (33.8)	36 (23.4)	
Fibrinogen concentration (g/L)			
≤ 3.47g/L	32 (20.8)	43 (27.9)	0.111
> 3.47 g/L	44 (28.6)	35 (22.7)	
FAR			
≤ 0.08	21 (13.6)	50 (32.5)	< 0.001
> 0.08	55 (35.7)	28 (18.2)	

FAR: Fibrinogen to albumin ratio.

Table 4 Correlation between FAR and clinicopathological characteristics in gallbladder cancer patients *n* (%)

Characteristics	FAR		<i>P</i> value
	≤ 0.08 (<i>n</i> = 71)	> 0.08 (<i>n</i> = 83)	
Age (yr)			
≤ 60	32 (20.8)	24 (15.6)	0.045
> 60	39 (25.3)	59 (38.3)	
Sex			
Male	30 (19.5)	33 (21.4)	0.870
Female	41 (26.6)	50 (32.5)	
Cholecystolithiasis			
Absent	37 (24.0)	42 (27.3)	0.873
Present	34 (22.1)	41 (26.6)	
Diabetes			
Absent	56 (36.4)	60 (39.0)	0.357
Present	15 (9.7)	23 (14.9)	
Jaundice			
Absent	67 (43.5)	62 (40.3)	< 0.001
Present	4 (2.6)	21 (13.6)	
Blood groups			
A	22 (14.3)	21 (13.6)	0.148
B	28 (18.2)	28 (18.2)	
AB	1 (0.6)	8 (5.2)	
O	20 (13.0)	26 (16.9)	
Pathological types			
Adenosquamous carcinoma	0 (0)	3 (1.9)	0.173
Adenocarcinoma	71 (46.1)	79 (51.3)	
Papilocarcinoma	0 (0)	1 (0.6)	
Degree of differentiation			
Poor	18 (11.7)	42 (27.3)	0.002
Moderate-well	53 (34.4)	41 (26.6)	
Resection margin status			
Negative	55 (35.7)	41 (26.6)	< 0.001
Positive	16 (10.4)	42 (27.3)	
Maximum tumor diameter (cm)			
≤ 2.45	37 (24.0)	31 (20.1)	0.075
> 2.45	34 (22.1)	52 (33.8)	
T stage			
Tis-T1a	8 (5.2)	2 (1.3)	< 0.001
T1b-T2b	24 (15.6)	5 (3.2)	
T3	36 (23.4)	67 (43.5)	
T4	3 (1.9)	9 (5.8)	
N stage			
N0	48 (31.2)	50 (32.5)	0.623
N1	19 (12.3)	28 (18.2)	
N2	4 (2.6)	5 (3.2)	
Distant metastasis			
Absent	68 (44.2)	74 (48.1)	0.145
Present	3 (1.9)	9 (5.8)	
TNM stage			
0- I stage	14 (9.1)	2 (1.3)	< 0.001
II A- II B stage	14 (9.1)	2 (1.3)	
III A- III B stage	35 (22.7)	57 (37.0)	
IV A- IV B stage	8 (5.2)	22 (14.3)	
CA199 (U/mL)			
≤ 39	43 (27.9)	23 (14.9)	< 0.001
> 39	28 (18.2)	60 (39.0)	
Fibrinogen concentration (g/L)			
≤ 3.47g/L	59 (38.3)	16 (10.4)	< 0.001
> 3.47 g/L	12 (7.8)	67 (43.5)	
Albumin levels (g/L)			
≤ 40.5g/L	21 (13.6)	55 (35.7)	< 0.001
> 40.5 g/L	50 (32.5)	28 (18.2)	

of GBC patients, such as resection margin status, TNM stage and albumin level, which were independent risk

factors for OS in GBC, indicating that an elevated FAR might be associated with aggressiveness and systemic progression of GBC.

Relatively few previous studies have probed into the prognostic significance of FAR in patients with malignant tumors. To date, there are only two studies performed in the context of breast cancer^[23] and ESCC^[22]. In line with our findings, the optimal cut-off value of FAR for ESCC patients was also 0.08. However, the optimal cut-off value of FAR in breast cancer was 0.071, which is slightly lower than that in ESCC patients and in GBC subjects in our study. Together, the inconsistent findings indicate that the optimal FAR cut-off value varies in different malignancies. Although the exact cause and underlying mechanism of these differences remain unknown, they might be related to the different biological behaviors of different tumors and gender-associated hormone difference. Hence, more studies are needed to further verify these conclusions.

Inconsistent with these previous two studies, our study indicates that the preoperative albumin level is also an independent risk factor for OS in GBC patients, and an elevated albumin level is a favorable prognostic factor for GBC patients. Several studies have demonstrated that lower serum albumin levels could lead to deteriorated diseases and a greater risk of poor prognosis in patients with gastric cancer^[26], ovarian cancer^[27] and upper urinary tract urothelial carcinoma^[12]. However, to our knowledge, it is the first study to assess the prognostic significance of preoperative serum albumin in GBC.

Accumulating studies have demonstrated the effect of activated coagulation with fibrinolysis, malnutrition and inflammation during carcinogenesis, cancer progression and metastasis^[28-31]. Although the prognostic value of the preoperative FAR has been established in patients with malignant tumors^[22,23], the real mechanisms underlying this association remains largely undefined. Our observations are supported by several previous experimental and clinical researches. As a P-globulin and pro-inflammatory protein, fibrinogen can be synthesized by malignant tumor cells apart from hepatic cells, which participates in extracellular matrix (ECM) formation^[32-34]. Fibrinogen can promote tumor progression via regulation of tumor cell growth by binding to vascular endothelial growth factor (VEGF) as well as platelet-derived growth factor (PDGF)^[33-35]. An experimental study has demonstrated that fibrinogen can induce epithelial-mesenchymal transition (EMT) to enhance the migration and invasion ability of tumor cells via modulation of the expression of vimentin and E-cadherin^[36]. Another experimental study performed in fibrinogen-deficient mice indicates that fibrinogen-free internal environment can suppress the spread of tumor cells and the subsequent establishment of micro-metastases^[37]. A previous study^[38] also showed that fibrinogen could facilitate tumor cell metastasis by suppressing natural killer (NK) cell-mediated apoptosis. Fibrinogen has

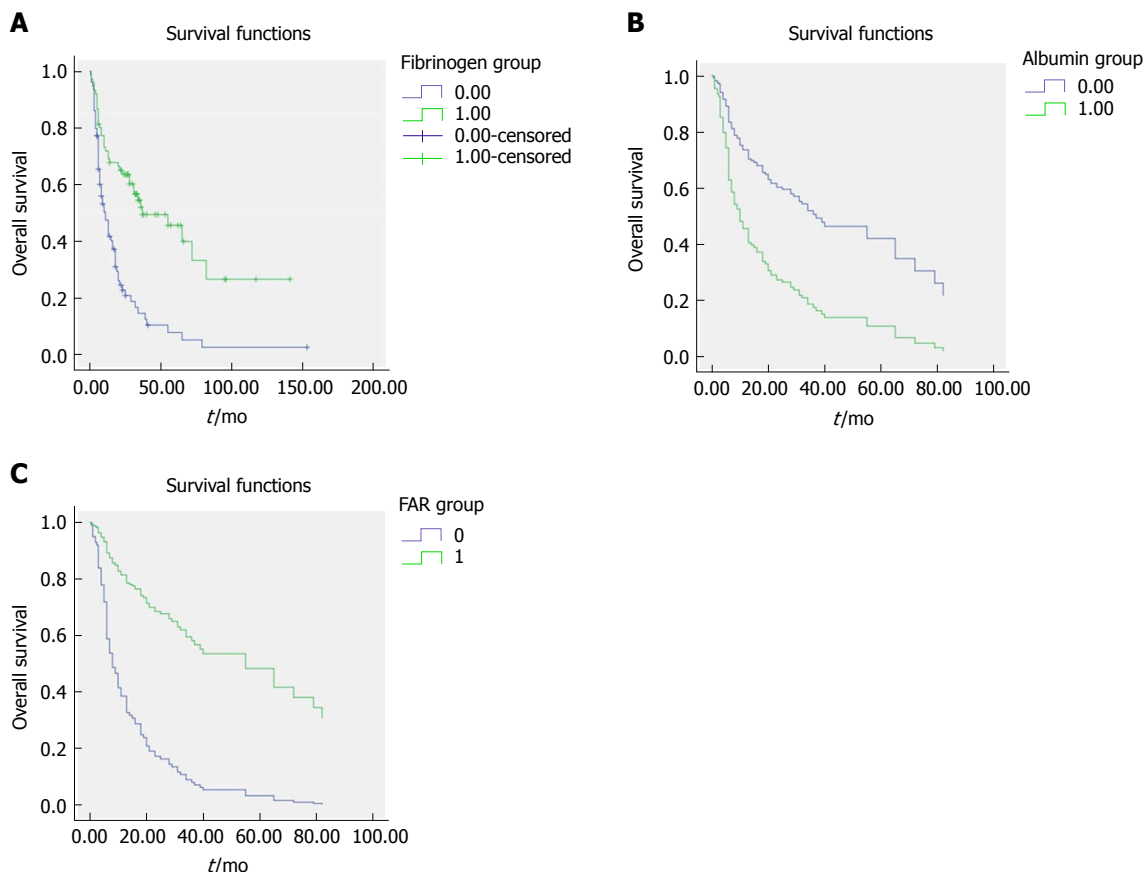


Figure 2 Survival curve according to the presence of preoperative fibrinogen concentration (A), albumin level (B), and fibrinogen to albumin ratio (C). A: Data compares fibrinogen concentration > 3.47 g/L vs ≤ 3.47 g/L group ($P < 0.05$). The number 1 for ≤ 3.47 g/L group, number 2 for > 3.47 g/L group; B: Data compares albumin level > 40.5 g/L vs ≤ 40.5 g/L group ($P < 0.05$). The number 0 for albumin level > 40.5 g/L group, number 1 for albumin level ≤ 40.5 g/L group; C: Data compares FAR > 0.08 vs ≤ 0.08 group ($P < 0.05$). The number 1 for FAR > 0.08 group, number 0 for FAR ≤ 0.08 g/L group. FAR: Fibrinogen to albumin ratio.

also been demonstrated to be critically involved in the tumorigenesis and tumor progression *via* aggravation of cell proliferation, suppression of apoptosis and stimulation of angiogenesis as well as hematogenous metastasis^[33,34,39-41]. The albumin level can not only reflect the malnutrition status of host, but also implicate the existence of inflammation. Malnutrition is commonly detected in cancer patients, which might lead to multiple negative outcomes, including compromised immune function, insensitive therapeutic response as well as reduced OS^[42]. As part of the SIR to tumor or from tumor itself, inflammatory mediators are secreted, including tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6 as well as acute-phase reactants. IL-6 has been suggested to modulate VEGF secretion from glioblastoma cells, and the latter can result in vascular permeability, contributing to declined serum albumin levels^[43,44]. Proinflammatory cytokines, such as TNF- α , IL-1 and IL-6 can downregulate the hepatic synthesis of albumin^[45-48]. Therefore, albumin level might be used to reflect tumor prognosis. Taken together, FAR could be considered as a prognostic factor for GBC patients.

There are certain limitations in this study. To begin with, this study was a retrospective, small-sample, single-center one, hence, there might be a selection

bias. Secondly, due to the small sample size, we were unable to perform further subgroup analysis according to different models, such as the TNM stage model, treatment model and distant metastasis model. Thirdly, our findings lacked external verification, which requires further investigation. Fourth, although the cut-off values were calculated by ROC curves, they were based on a relatively small sample; as such, other cut-off values may be more accurate in the case of increased sample size. In this study, we mainly focused on the prognostic significance of the preoperative FAR, while changes in the postoperative FAR have not been studied; thus, the prognostic value of the postoperative FAR was not assessed. Therefore, more well-designed, prospective and large-sample multi-center studies are warranted to further verify the present conclusions.

In conclusion, the preoperative FAR is a significant and powerful negative prognostic indicator for OS in GBC patients, and the preoperative serum albumin level is a favorable prognostic factor for OS in GBC patients, and the predictive power of FAR is greater than that of the albumin level. As a simple, convenient and cost-effective indicator, FAR, defined as the fibrinogen-to-albumin ratio, could easily be applied in the clinical setting via routine preoperative laboratory tests to predict the

Table 5 Univariate analysis of overall survival in gallbladder cancer patients

Characteristics	HR (95%CI)	P value
Age (yr)	1.473 (0.973-2.230)	0.067
≤ 60		
> 60		
Sex	0.995 (0.670-1.477)	0.981
Male		
Female		
Cholecystolithiasis	1.198 (0.814-1.764)	0.360
Absent		
Present		
Diabetes	1.028 (0.651-1.623)	0.906
Absent		
Present		
Jaundice	2.598 (1.644-4.106)	< 0.001
Absent		
Present		
Blood groups	-	0.113
A		
B		
AB		
O		
Pathological types	-	0.165
Adenosquamous carcinoma		
Adenocarcinoma		
Papilocarcinoma		
Degree of differentiation	1.527 (1.031-2.261)	0.035
Poor		
Moderate-well		
Resection margin status	3.683 (2.468-5.496)	< 0.001
Negative		
Positive		
Maximum tumor diameter (cm)	1.101 (0.744-1.630)	0.631
≤ 2.45		
> 2.45		
T stage	-	< 0.001
Tis-T1a		
T1b-T2b		
T3		
T4		
N stage	-	< 0.001
0		
1		
2		
Distant metastasis	2.550 (1.388-4.684)	0.003
Absent		
Present		
TNM stage	-	< 0.001
0- I stage		
II A- II B stage		
III A-III B stage		
IV A-IV B stage		
CA199 (U/mL)	3.125 (2.010-4.858)	< 0.001
≤ 39		
> 39		
Fibrinogen concentration (g/L)	2.795 (1.853-4.214)	< 0.001
≤ 3.47		
> 3.47		
Albumin levels (g/L)	0.391(0.259-0.590)	< 0.001
≤ 40.5		
> 40.5		
FAR	4.626(2.987-7.165)	< 0.001
≤ 0.08		
> 0.08		

FAR: Fibrinogen to albumin ratio; HR: Hazard ratio; CI: Confidence interval.

Table 6 Multivariate analysis for overall survival in gallbladder cancer patients

Characteristics	HR (95%CI)	Wald	P value
Resection margin status	2.343 (1.532-3.581)		< 0.001
Negative			
Positive			
TNM stage		8.595	0.035
II A- II B stage/0-1stage	1.209 (0.287-5.095)	0.067	0.796
III A-III B stage/0-1 stage	3.401 (1.033-11.202)	4.051	0.044
IV A-IV B stage/0-1 stage	4.014 (1.142-14.107)	4.696	0.030
FAR	2.813 (1.765-4.484)		< 0.001
≤ 0.08			
> 0.08			
Albumin levels (g/L)	0.595 (0.385-0.921)		0.020
≤ 40.5			
> 40.5			

FAR: Fibrinogen to albumin ratio; HR: Hazard ratio; CI: Confidence interval.

prognosis of GBC patients. However, more related studies are warranted to validate these conclusions.

ARTICLE HIGHLIGHTS

Research background

Although gallbladder cancer (GBC) is a relatively rare hepato-biliary malignancy with a low incidence, it is generally insidious and progresses rapidly. Most GBC patients are diagnosed at an advanced stage, losing the chance of surgical intervention, which is considered to yield an optimal therapeutic effect. Despite the great advance in surgical techniques in recent years, the prognosis remains very poor. Therefore, it is urgent to explore a clinically simple, convenient and cost-effective prognostic indicator to detect and identify high-risk patients with GBC, on whom, appropriate surgical treatment can be performed as soon as possible. In recent years, a variety of studies have shown that the increased plasma fibrinogen concentration representing coagulation function of the body and the declined plasma albumin concentration indicating nutrient state of the body are independent risk factors for poor prognosis of malignant tumor patients. Integrating the results of studies on fibrinogen-to-albumin ratio (FAR) in the prognosis of patients with esophageal cancer and breast cancer, we naturally speculate that FAR might be significantly more effective than single elevated plasma fibrinogen concentration or reduced plasma albumin concentration in predicting the prognosis of GBC patients.

Research motivation

Hence, the present was mainly designed to determine and verify the role of high FAR in the prognosis of surgically-treated GBC patients. We aimed to detect a simple, convenient and cost-effective prognostic biomarker for GBC patients undergoing surgical treatment, which could facilitate the selection and identification of GBC patients suitable for surgical resection for clinical surgeons. Notably, this would be beneficial to both surgeons and GBC patients. Our findings would provide clinical evidence and research directions for other large-scale, multi-center randomized controlled trials in the future.

Research objectives

The main objective of our study was to determine whether high preoperative FAR was an independent risk factors for postoperative survival in GBC patients. As a result, we demonstrated that high preoperative plasma FAR value and low preoperative plasma albumin concentration were independent risk factors for poor post-operative prognosis of GBC patients. In addition, the prognostic effect of high preoperative FAR value was significantly stronger than the low preoperative plasma albumin concentration. Therefore, these above-described outcomes provided not only clinical direction for further clinical validation or relevant studies, but also clinical data for further researches concerning the

underlying mechanisms.

Research methods

First, the present study was a clinical retrospective one. A prearranged EXCEL data collection table was utilized to collect and organize the various variables, including epidemiological data, clinicopathological characteristics, and research-related target data. Moreover, the receiver operating characteristic (ROC) curve was used to obtain the optimal cut-off values for fibrinogen, albumin, and FAR. Continuous variables in normal distribution were shown as mean \pm SD, and continuous variables without normal distribution were expressed as medians (range: minimum-maximum). Categorical variables were expressed as percentages or frequencies. Variables from the EXCEL table were further imported into the SPSS 24.0 statistical software for statistical analysis. Of note, the statistical methods used in our study were different from those used in previous studies of survival analysis regarding the prognosis of cancer patients. To begin with, the ROC curve was used to identify the optimal cut-off value of fibrinogen, albumin, and FAR in this study, which was more reasonable and more scientific than the traditional methods, which used the mean value of the targeted or identified biomarkers based on previous studies. It was because the cut-off value identified by this method was significantly associated with the overall survival of the targeted population. Secondly, most of the previous studies on postoperative prognosis of GBC patients only focused on single index, such as plasma fibrinogen or plasma albumin. However, in this study, we used the plasma FAR, representing the division of high fibrinogen and low albumin, which contributed to the more significant prognostic effect of the index, and effectively inhibited the influence of confounding factors. Together, the method was more scientific and harbored higher statistical efficiency.

Research results

In this study, we demonstrated that high preoperative plasma FAR and low preoperative plasma albumin concentration were independent risk factors for poor postoperative outcome in GBC patients. To the best of our knowledge, this is the first study indicating that high preoperative plasma FAR is an unfavorable prognostic biomarker for GBC patients undergoing surgical intervention. Additionally, it also verifies the role of low preoperative plasma albumin in predicting the worse prognosis of GBC patients receiving surgery. Nevertheless, our study is a retrospective study but not a prospective study, which might lead to a systematic bias. Moreover, the sample size in our study is relatively small, and it is a single-center study, and these defects would attenuate the statistical effectiveness of our conclusions. Nevertheless, the study was conducted in China, which did not include GBC patients from other ethnic groups and countries, thereby affecting the clinical applicability and generalizability of the results. Therefore, more multiple-center, large-scale prospective studies enrolling GBC patients from different races and countries are necessary to further verify the conclusions of this study.

Research conclusions

At present, accumulating studies have confirmed that high preoperative plasma fibrinogen concentration and low preoperative plasma albumin concentration are independent risk factors for poor prognosis of GBC patients. In addition, some studies have further validated that high preoperative plasma FAR is an independent risk factor for poor prognosis of patients with esophageal cancer and breast cancer, and its predictive ability is significantly more potent than that of single biomarkers, such as high plasma fibrinogen and low plasma albumin. Therefore, we naturally speculated that FAR, representing the body's coagulation function and the body's nutritional status, might be an independent risk factor for predicting postoperative adverse outcomes of GBC patients, which has been confirmed in our study. Our study was the first to reveal the prognostic effect of FAR in GBC patients, and we also used the ROC curve as a novel method to identify the optimal cut-off value for the prognostic index studied. The potential mechanism for our conclusion might be indicated as follows: fibrinogen, as a coagulation factor, was associated with the growth, progression and metastasis of cancer cells, while albumin was correlated with the nutrient status and immune function of the body. Therefore, the high preoperative plasma fibrinogen and low preoperative albumin are both unfavorable prognostic factors for GBC patients. The FAR can enhance and magnify the prognostic effect of the single index such as fibrinogen and albumin. Collectively, our research provides a simple, convenient and cost-effective prognostic indicator to help clinicians to more efficiently screen and

identify high-risk GBC patients in clinical practice, and to facilitate patients to adopt better surgical methods and optimal follow-up strategy in the future.

Research perspectives

In the present study, it is indicated that the plasma FAR, incorporating two biomarkers, harbors a significantly better prognostic impact on surgically-treated GBC patients compared to a single prognostic indicator, such as plasma albumin or plasma fibrinogen. In the future, more large-scale, multiple-center and prospective studies, including GBC patients from other races and countries, should be conducted to further investigate and verify the conclusion derived from our study. Additionally, more basic experiments exploring the potential mechanisms are also necessary in the future.

REFERENCES

- 1 **Zhu AX**, Hong TS, Hezel AF, Kooby DA. Current management of gallbladder carcinoma. *Oncologist* 2010; **15**: 168-181 [PMID: 20147507 DOI: 10.1634/theoncologist.2009-0302]
- 2 **Paliogiannis P**, Scognamiglio F, Attene F, Marrosu A, Trignano E, Tedde L, Delogu D, Trignano M. Preneoplastic and neoplastic gallbladder lesions occasionally discovered after elective videocholecystectomy for benign disease. A single centre experience and literature review. *Ann Ital Chir* 2013; **84**: 281-285 [PMID: 23047610]
- 3 **Misra S**, Chaturvedi A, Misra NC, Sharma ID. Carcinoma of the gallbladder. *Lancet Oncol* 2003; **4**: 167-176 [PMID: 12623362 DOI: 10.1016/S1470-2045(03)01021-0]
- 4 **Duffy A**, Capanu M, Abou-Alfa GK, Huitzil D, Jarnagin W, Fong Y, D'Angelica M, Dematteo RP, Blumgart LH, O'Reilly EM. Gallbladder cancer (GBC): 10-year experience at Memorial Sloan-Kettering Cancer Centre (MSKCC). *J Surg Oncol* 2008; **98**: 485-489 [PMID: 18802958 DOI: 10.1002/jso.21141]
- 5 **Lai EC**, Lau WY. Aggressive surgical resection for carcinoma of gallbladder. *ANZ J Surg* 2005; **75**: 441-444 [PMID: 15943734 DOI: 10.1111/j.1445-2197.2005.03401.x]
- 6 **Bartlett DL**, Fong Y, Fortner JG, Brennan MF, Blumgart LH. Long-term results after resection for gallbladder cancer. Implications for staging and management. *Ann Surg* 1996; **224**: 639-646 [PMID: 8916879]
- 7 **Forrest LM**, McMillan DC, McArdle CS, Angerson WJ, Dunlop DJ. Comparison of an inflammation-based prognostic score (GPS) with performance status (ECOG) in patients receiving platinum-based chemotherapy for inoperable non-small-cell lung cancer. *Br J Cancer* 2004; **90**: 1704-1706 [PMID: 15150622 DOI: 10.1038/sj.bjc.6601789]
- 8 **Ahn HS**, Lee HJ, Yoo MW, Kim SG, Im JP, Kim SH, Kim WH, Lee KU, Yang HK. Diagnostic accuracy of T and N stages with endoscopy, stomach protocol CT, and endoscopic ultrasonography in early gastric cancer. *J Surg Oncol* 2009; **99**: 20-27 [PMID: 18937292 DOI: 10.1002/jso.21170]
- 9 **Lee HH**, Lim CH, Park JM, Cho YK, Song KY, Jeon HM, Park CH. Low accuracy of endoscopic ultrasonography for detailed T staging in gastric cancer. *World J Surg Oncol* 2012; **10**: 190 [PMID: 22978534 DOI: 10.1186/1477-7819-10-190]
- 10 **Ando M**, Ando Y, Hasegawa Y, Shimokata K, Minami H, Wakai K, Ohno Y, Sakai S. Prognostic value of performance status assessed by patients themselves, nurses, and oncologists in advanced non-small cell lung cancer. *Br J Cancer* 2001; **85**: 1634-1639 [PMID: 11742480 DOI: 10.1054/bjoc.2001.2162]
- 11 **Rajan R**, Poniecka A, Smith TL, Yang Y, Frye D, Pusztai L, Fitterman DJ, Gal-Gombos E, Whitman G, Rouzier R, Green M, Kuerer H, Buzdar AU, Hortobagyi GN, Symmans WF. Change in tumor cellularity of breast carcinoma after neoadjuvant chemotherapy as a variable in the pathologic assessment of response. *Cancer* 2004; **100**: 1365-1373 [PMID: 15042669 DOI: 10.1002/cncr.20134]
- 12 **Ku JH**, Kim M, Choi WS, Kwak C, Kim HH. Preoperative serum albumin as a prognostic factor in patients with upper urinary tract urothelial carcinoma. *Int Braz J Urol* 2014; **40**: 753-762 [PMID:

- 25615244 DOI: 10.1590/S1677-5538.IBJU.2014.06.06]
- 13 **Perisanidis C**, Psyrri A, Cohen EE, Engelmann J, Heinze G, Perisanidis B, Stift A, Filipits M, Kornek G, Nkenke E. Prognostic role of pretreatment plasma fibrinogen in patients with solid tumors: A systematic review and meta-analysis. *Cancer Treat Rev* 2015; **41**: 960-970 [PMID: 26604093 DOI: 10.1016/j.ctrv.2015.10.002]
- 14 **Son HJ**, Park JW, Chang HJ, Kim DY, Kim BC, Kim SY, Park SC, Choi HS, Oh JH. Preoperative plasma hyperfibrinogenemia is predictive of poor prognosis in patients with nonmetastatic colon cancer. *Ann Surg Oncol* 2013; **20**: 2908-2913 [PMID: 23612884 DOI: 10.1245/s10434-013-2968-8]
- 15 **Bairey O**, Shacham-Abulafia A, Shpilberg O, Gurion R. Serum albumin level at diagnosis of diffuse large B-cell lymphoma: an important simple prognostic factor. *Hematol Oncol* 2016; **34**: 184-192 [PMID: 26052918 DOI: 10.1002/hon.2233]
- 16 **Gupta D**, Lis CG. Pretreatment serum albumin as a predictor of cancer survival: a systematic review of the epidemiological literature. *Nutr J* 2010; **9**: 69 [PMID: 21176210 DOI: 10.1186/1475-2891-9-69]
- 17 **Lee JH**, Hyun JH, Kim DY, Yoo BC, Park JW, Kim SY, Chang HJ, Kim BC, Kim TH, Oh JH, Sohn DK. The role of fibrinogen as a predictor in preoperative chemoradiation for rectal cancer. *Ann Surg Oncol* 2015; **22**: 209-215 [PMID: 25384698 DOI: 10.1245/s10434-014-3962-5]
- 18 **Zhao J**, Zhao M, Jin B, Yu P, Hu X, Teng Y, Zhang J, Luo Y, Zhang L, Zheng S, Zhou Q, Li H, Liu Y, Qu X. Tumor response and survival in patients with advanced non-small-cell lung cancer: the predictive value of chemotherapy-induced changes in fibrinogen. *BMC Cancer* 2012; **12**: 330 [PMID: 22852778 DOI: 10.1186/1471-2407-12-330]
- 19 **Pichler M**, Hutterer GC, Stojakovic T, Mannweiler S, Pummer K, Zigeuner R. High plasma fibrinogen level represents an independent negative prognostic factor regarding cancer-specific, metastasis-free, as well as overall survival in a European cohort of non-metastatic renal cell carcinoma patients. *Br J Cancer* 2013; **109**: 1123-1129 [PMID: 23922109 DOI: 10.1038/bjc.2013.443]
- 20 **Wen J**, Yang Y, Ye F, Huang X, Li S, Wang Q, Xie X. The preoperative plasma fibrinogen level is an independent prognostic factor for overall survival of breast cancer patients who underwent surgical treatment. *Breast* 2015; **24**: 745-750 [PMID: 26482138 DOI: 10.1016/j.breast.2015.09.007]
- 21 **Borg N**, Guilfoyle MR, Greenberg DC, Watts C, Thomson S. Serum albumin and survival in glioblastoma multiforme. *J Neurooncol* 2011; **105**: 77-81 [PMID: 21409514 DOI: 10.1007/s11060-011-0562-0]
- 22 **Tan Z**, Zhang M, Han Q, Wen J, Luo K, Lin P, Zhang L, Yang H, Fu J. A novel blood tool of cancer prognosis in esophageal squamous cell carcinoma: the Fibrinogen/Albumin Ratio. *J Cancer* 2017; **8**: 1025-1029 [PMID: 28529615 DOI: 10.7150/jca.16491]
- 23 **Hwang KT**, Chung JK, Roh EY, Kim J, Oh S, Kim YA, Rhu J, Kim S. Prognostic Influence of Preoperative Fibrinogen to Albumin Ratio for Breast Cancer. *J Breast Cancer* 2017; **20**: 254-263 [PMID: 28970851 DOI: 10.4048/jbc.2017.20.3.254]
- 24 **General Assembly of the World Medical Association**. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *J Am Coll Dent* 2014; **81**: 14-18 [PMID: 25951678]
- 25 **CLAUSS A**. [Rapid physiological coagulation method in determination of fibrinogen]. *Acta Haematol* 1957; **17**: 237-246 [PMID: 13434757 DOI: 10.1159/000205234]
- 26 **Crumley AB**, Stuart RC, McKernan M, McMillan DC. Is hypoalbuminemia an independent prognostic factor in patients with gastric cancer? *World J Surg* 2010; **34**: 2393-2398 [PMID: 20602101 DOI: 10.1007/s00268-010-0641-y]
- 27 **Asher V**, Lee J, Bali A. Preoperative serum albumin is an independent prognostic predictor of survival in ovarian cancer. *Med Oncol* 2012; **29**: 2005-2009 [PMID: 21735143 DOI: 10.1007/s12032-011-0019-5]
- 28 **Unsal E**, Atalay F, Atikcan S, Yilmaz A. Prognostic significance of hemostatic parameters in patients with lung cancer. *Respir Med* 2004; **98**: 93-98 [PMID: 14971870 DOI: 10.1016/j.rmed.2003.07.001]
- 29 **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]
- 30 **Sun KY**, Xu JB, Chen SL, Yuan YJ, Wu H, Peng JJ, Chen CQ, Guo P, Hao YT, He YL. Novel immunological and nutritional-based prognostic index for gastric cancer. *World J Gastroenterol* 2015; **21**: 5961-5971 [PMID: 26019461 DOI: 10.3748/wjg.v21.i19.5961]
- 31 **Zitvogel L**, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* 2006; **6**: 715-727 [PMID: 16977338 DOI: 10.1038/nri1936]
- 32 **Simpson-Haidaris PJ**, Rybarczyk B. Tumors and fibrinogen. The role of fibrinogen as an extracellular matrix protein. *Ann N Y Acad Sci* 2001; **936**: 406-425 [PMID: 11460495 DOI: 10.1111/j.1749-6632.2001.tb03525.x]
- 33 **Sahni A**, Khorana AA, Baggs RB, Peng H, Francis CW. FGF-2 binding to fibrin(ogen) is required for augmented angiogenesis. *Blood* 2006; **107**: 126-131 [PMID: 16160009 DOI: 10.1182/blood-2005-06-2460]
- 34 **Sahni A**, Simpson-Haidaris PJ, Sahni SK, Vaday GG, Francis CW. Fibrinogen synthesized by cancer cells augments the proliferative effect of fibroblast growth factor-2 (FGF-2). *J Thromb Haemost* 2008; **6**: 176-183 [PMID: 17949478 DOI: 10.1111/j.1538-7836.2007.02808.x]
- 35 **Witsch E**, Sela M, Yarden Y. Roles for growth factors in cancer progression. *Physiology* (Bethesda) 2010; **25**: 85-101 [PMID: 20430953 DOI: 10.1152/physiol.00045.2009]
- 36 **Shu YJ**, Weng H, Bao RF, Wu XS, Ding Q, Cao Y, Wang XA, Zhang F, Xiang SS, Li HF, Li ML, Mu JS, Wu WG, Liu YB. Clinical and prognostic significance of preoperative plasma hyperfibrinogenemia in gallbladder cancer patients following surgical resection: a retrospective and in vitro study. *BMC Cancer* 2014; **14**: 566 [PMID: 25096189 DOI: 10.1186/1471-2407-14-566]
- 37 **Palumbo JS**, Potter JM, Kaplan LS, Talmage K, Jackson DG, Degen JL. Spontaneous hematogenous and lymphatic metastasis, but not primary tumor growth or angiogenesis, is diminished in fibrinogen-deficient mice. *Cancer Res* 2002; **62**: 6966-6972 [PMID: 12460914]
- 38 **Zheng S**, Shen J, Jiao Y, Liu Y, Zhang C, Wei M, Hao S, Zeng X. Platelets and fibrinogen facilitate each other in protecting tumor cells from natural killer cytotoxicity. *Cancer Sci* 2009; **100**: 859-865 [PMID: 19302289 DOI: 10.1111/j.1349-7006.2009.01115.x]
- 39 **Steinbrecher KA**, Horowitz NA, Blevins EA, Barney KA, Shaw MA, Harmel-Laws E, Finkelman FD, Flick MJ, Pinkerton MD, Talmage KE, Kombrinck KW, Witte DP, Palumbo JS. Colitis-associated cancer is dependent on the interplay between the hemostatic and inflammatory systems and supported by integrin alpha(M)beta(2) engagement of fibrinogen. *Cancer Res* 2010; **70**: 2634-2643 [PMID: 20233870 DOI: 10.1158/0008-5472.CAN-09-3465]
- 40 **Martino MM**, Briquez PS, Ranga A, Lutolf MP, Hubbell JA. Heparin-binding domain of fibrin(ogen) binds growth factors and promotes tissue repair when incorporated within a synthetic matrix. *Proc Natl Acad Sci USA* 2013; **110**: 4563-4568 [PMID: 23487783 DOI: 10.1073/pnas.1221602110]
- 41 **Staton CA**, Brown NJ, Lewis CE. The role of fibrinogen and related fragments in tumour angiogenesis and metastasis. *Expert Opin Biol Ther* 2003; **3**: 1105-1120 [PMID: 14519075 DOI: 10.1517/14712598.3.7.1105]
- 42 **Van Cutsem E**, Arends J. The causes and consequences of cancer-associated malnutrition. *Eur J Oncol Nurs* 2005; **9** Suppl 2: S51-S63 [PMID: 16437758 DOI: 10.1016/j.ejon.2005.09.007]
- 43 **Fleck A**, Raines G, Hawker F, Trotter J, Wallace PI, Ledingham IM, Calman KC. Increased vascular permeability: a major cause of hypoalbuminaemia in disease and injury. *Lancet* 1985; **1**: 781-784 [PMID: 2858667 DOI: 10.1016/S0140-6736(85)91447-3]
- 44 **Loeffler S**, Fayard B, Weis J, Weissenberger J. Interleukin-6 induces transcriptional activation of vascular endothelial growth

- factor (VEGF) in astrocytes in vivo and regulates VEGF promoter activity in glioblastoma cells via direct interaction between STAT3 and Sp1. *Int J Cancer* 2005; **115**: 202-213 [PMID: 15688401 DOI: 10.1002/ijc.20871]
- 45 **Rothschild MA**, Oratz M, Schreiber SS. Serum albumin. *Hepatology* 1988; **8**: 385-401 [PMID: 3281888 DOI: 10.1002/hep.1840080234]
 - 46 **Deehan DJ**, Heys SD, Simpson W, Herriot R, Broom J, Eremin O. Correlation of serum cytokine and acute phase reactant levels with alterations in weight and serum albumin in patients receiving immunotherapy with recombinant IL-2. *Clin Exp Immunol* 1994; **95**: 366-372 [PMID: 7511074 DOI: 10.1111/j.1365-2249.1994.tb07005.x]
 - 47 **Nakashima J**, Tachibana M, Ueno M, Miyajima A, Baba S, Murai M. Association between tumor necrosis factor in serum and cachexia in patients with prostate cancer. *Clin Cancer Res* 1998; **4**: 1743-1748 [PMID: 9676850]
 - 48 **Mantovani A**, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008; **454**: 436-444 [PMID: 18650914 DOI: 10.1038/nature07205]

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Observational Study

Fatigue is not associated with vitamin D deficiency in inflammatory bowel disease patients

Svein Oskar Frigstad, Marte Lie Høivik, Jørgen Jahnsen, Milada Cvancarova, Tore Grimstad, Ingrid Prytz Berset, Gert Huppertz-Hauss, Øistein Hovde, Tømm Bernklev, Bjørn Moum, Lars-Petter Jelsness-Jørgensen

Svein Oskar Frigstad, Department of Medicine, Vestre Viken Bærum Hospital, Gjøttum 1346, Norway

Svein Oskar Frigstad, Department of Research, Østfold Hospital Kalnes, Grålum 1714, Norway

Marte Lie Høivik, Bjørn Moum, Department of Gastroenterology, Oslo University Hospital, Oslo 0424, Norway

Jørgen Jahnsen, Department of Gastroenterology, Akershus University Hospital, Lørenskog 1478, Norway

Milada Cvancarova, Faculty of Health Sciences, Oslo Metropolitan University, Oslo 0130, Norway

Tore Grimstad, Department of Gastroenterology, Stavanger University Hospital, Stavanger 4068, Norway

Ingrid Prytz Berset, Department of Medicine, Ålesund Hospital Trust, Ålesund 6026, Norway

Gert Huppertz-Hauss, Department of Gastroenterology, Telemark Hospital Trust, Skien 3710, Norway

Øistein Hovde, Department of Medicine, Innlandet Hospital Trust, Gjøvik 2819, Norway

Tømm Bernklev, Department of Research and Development, Vestfold Hospital Trust, Tønsberg 3103, Norway

Lars-Petter Jelsness-Jørgensen, Department of Gastroenterology, Østfold Hospital Kalnes, Grålum 1714, Norway

Lars-Petter Jelsness-Jørgensen, Department of Health Sciences, Østfold University College, Halden 1757, Norway

ORCID number: Svein Oskar Frigstad (0000-0001-7841-608X); Marte Lie Høivik (0000-0002-0104-465X); Jørgen Jahnsen (0000-0002-0419-2914); Milada Cvancarova (0000-0001-8947-8649); Tore Grimstad (0000-0001-6669-5843); Ingrid Prytz Berset (0000-0002-2014-8497); Gert Huppertz-Hauss (0000-0002-5693-8773); Øistein Hovde (0000-0003-4662-4153); Tømm Bernklev (0000-0002-9988-2271); Bjørn Moum

(0000-0002-5884-4543); Lars-Petter Jelsness-Jørgensen (0000-0002-5465-1576).

Author contributions: Frigstad SO performed the study, analysed the data and wrote the paper; Høivik ML and Cvancarova M analyzed the data, contributed to the design of tables and writing of the paper; Jahnsen J performed the study, contributed to tables and writing of the paper; Grimstad T, Berset IP, Huppertz-Hauss G and Hovde Ø performed the study and contributed to writing of the paper; Bernklev T contributed to the design of the study and writing of the paper; Moum B contributed to the design of the study, performed the study and supervised the writing of the paper; Jelsness-Jørgensen LP designed, supervised and performed the study, contributed to analysing the data and revised the paper; all authors have approved the final manuscript.

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Correspondence to: Svein Oskar Frigstad, MD, Doctor, Research Fellow, Department of Research, Østfold Hospital Kalnes, Postboks 300, Grålum 1714, Norway. s.o.frigstad@medisin.uio.no
Telephone: +47-91508600
Fax: +47-69863285

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Abstract

AIM

To investigate if vitamin D deficiency is associated with fatigue in patients with inflammatory bowel disease (IBD).

METHODS

IBD patients were recruited from nine hospitals in the southeastern and western regions of Norway to participate in a multicenter cross-sectional study lasting from March 2013 to April 2014. Data were collected by interviews, from medical records and laboratory tests. The Fatigue Questionnaire (FQ) was used to measure fatigue. Linear and logistic regression models were applied to explore the possible association between vitamin D deficiency and total fatigue scores and chronic fatigue, respectively. The analyses were adjusted for age, gender, disease activity, depressive symptoms and sleep disturbance.

RESULTS

In total, 405 patients were included in the analyses, of which 227 (56%) had Crohn's disease (CD) and 178 (44%) had ulcerative colitis (UC). Vitamin D deficiency (< 50 nmol/L) was present in half (203/405) of the patients. Chronic fatigue was reported by 116 (29%) of all included patients with substantial fatigue reported by 194 (48%). Vitamin D levels were neither associated with total fatigue nor with chronic fatigue. Higher total fatigue scores and chronic fatigue were both associated with increased disease activity scores in patients with UC and CD, but not with increased CRP or fecal calprotectin. In UC patients, female gender was associated with fatigue in the univariate analysis, but no such difference was found when adjusted for elevated disease activity scores. Sleep disturbance and more depressive symptoms were associated with total fatigue scores in both UC and CD patients, but with chronic fatigue only in CD patients.

CONCLUSION

In this study, no significant association between fatigue and vitamin D deficiency in IBD patients was revealed.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Fatigue; Chronic fatigue; Vitamin D deficiency

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Core tip: Fatigue is common in inflammatory bowel diseases (IBD) and is especially prevalent in active disease. We wanted to investigate if vitamin D deficiency was associated to fatigue in IBD as this is a common belief among both patients and physicians. To the best of our knowledge, no previous studies have investigated this possible association in IBD patients. We also wanted to take into account the multidimensional approach in understanding fatigue making it difficult to single out any one contributing factor. In this study, no significant association between fatigue and vitamin D deficiency in IBD patients was revealed.

Frigstad SO, Høivik ML, Jahnsen J, Cvancarova M, Grimstad T, Berset IP, Huppertz-Hauss G, Hovde Ø, Bernklev T, Moum B, Jelsness-Jørgensen LP. Fatigue is not associated with vitamin D deficiency in inflammatory bowel disease patients. *World J Gastroenterol* 2018; 24(29): 3293-3301 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i29/3293.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i29.3293>

INTRODUCTION

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are characterized by chronic, recurrent inflammation of the gastrointestinal tract^[1-3]. IBD may present a variety of symptoms such as diarrhea with or without blood, abdominal pain, and fatigue. Patients with IBD often report the latter as one of their most troublesome symptoms^[4].

Fatigue is common in IBD, with a prevalence of 44%-86% in active disease and 22%-41% in remission^[5-7]. The patients usually report an overwhelming feeling of tiredness, reduced energy levels, reduced muscle strength, and cognitive impairment, which supports a multidimensional approach to understanding and measuring fatigue^[5,6,8,9]. Chronic fatigue has been defined as severe fatigue persisting for at least six months leading to the substantial impairment of daily life^[10,11].

Fatigue is associated with reduced health-related quality of life (HRQoL) and work ability^[4,10,12-15]. In IBD, factors such as disease activity, disturbed sleep, anemia, pain and depression have all been associated with fatigue^[5,6,10,16-19]. Anemia and pain are both related to more active disease, and nocturnal symptoms in IBD may be associated with sleep disturbance, a

known factor predisposing to fatigue. In addition, patients with severe fatigue are more likely to suffer from mood disorders such as depression, and there is an association between fatigue and psychological comorbidity^[20].

Vitamin D deficiency has been reported to be associated with fatigue in cancer patients^[21]. Moreover, vitamin D supplementation has been shown to be significantly associated with the improvement of fatigue symptoms in other chronic inflammatory conditions^[22,23]. Several studies have shown that vitamin D deficiency is common in patients with IBD, that it is seen more often in patients with CD than in those with UC and that it is more frequent in these patients than in the general population^[24-29].

Furthermore, vitamin D deficiency has been associated with disease activity in IBD patients^[24,27,28,30-32]. Low vitamin D levels in IBD may result in increased fatigue that is partly due to more active disease. However, fatigue often persists after the patient has achieved clinical remission, without any objectively measured signs of intestinal inflammatory activity. This indicates that fatigue is not only a symptom related to inflammation^[5].

The high prevalence of vitamin D deficiency may also be of potential importance in fatigue, as muscle weakness has been linked to vitamin D deficiency^[33]. Skeletal muscle has vitamin D receptors and may require vitamin D for maximal function, and thus vitamin D deficiency may result in physical fatigue, one of the dimensions frequently measured in the assessment of fatigue^[33].

The aim of this study was to investigate the possible association between vitamin D deficiency and fatigue in patients with IBD.

MATERIALS AND METHODS

Study area and population

Patients were recruited from nine hospitals in the southeastern and western part of Norway as part of an observational, multicenter cross-sectional study. Inclusion criteria were: Age ≥ 18 years, a verified diagnosis of IBD based on endoscopic, biochemical and histological findings according to the Lennard-Jones criteria^[34], ability to read and understand Norwegian and to give written informed consent. The inclusion period lasted from March 2013 to April 2014. At each of the included centers, a senior gastroenterologist was responsible for the study.

Clinical, socio-demographic and laboratory variables

Data were collected by interviews, from medical records and laboratory tests. All data were collected at inclusion.

Serum 25-OH-D from all patients was analyzed by one central accredited laboratory with liquid chromatography tandem mass spectrometry (LC-MS/MS), and the method used for vitamin D analysis has

been compared to the LC-MS/MS method used in the vitamin D standardizing Program VDSP and showed good compliance^[35]. Vitamin D deficiency was defined as 25-OH-D concentration < 50 nmol/L, and vitamin D insufficiency was defined as a 25-OH-D concentration of 50-75 nmol/L^[33].

All other biochemical analyses were performed at the local laboratories at the participating centers. C-reactive protein (CRP) levels of 5 mg/L or higher were chosen to indicate active inflammation^[36,37].

The stool samples for the measurement of fecal calprotectin were sent by mail and analyzed with Calpro Easy Extract (Calpro AS, Norway). Fecal calprotectin < 100 mg/kg was set as the cut-off for disease in remission, while higher levels were defined as active inflammation^[38].

Clinical disease activity was measured with the Simple Clinical Colitis Activity Index (SCCAI) for UC and the Harvey Bradshaw Index (HBI) for CD^[39,40]. For UC, an SCCAI score ≥ 5 was defined as active disease^[36,37,41,42], and in CD, an HBI score ≥ 5 was defined as active disease^[39,43].

Assessment of fatigue, depressive symptoms and quality of sleep

Fatigue was measured with the Fatigue Questionnaire (FQ)^[8,44]. The FQ has been found to be suitable both for clinical and epidemiological purposes and has been translated into Norwegian and validated^[8]. Two dimensions of fatigue are measured, physical fatigue (items 1-7) and mental fatigue (items 8-11). The sum of all items produces the total fatigue score. Each item score can be dichotomized (0 to 1 = 0, 2 to 3 = 1), giving a total score between 0 and 11. Chronic fatigue (CF) was defined as dichotomized FQ scores ≥ 4 with a duration > 6 mo, in accordance with previous studies^[8,10,44,45].

The presence of depressive symptoms was measured with the Hospital Anxiety and Depression Scale (HADS), which has been translated into Norwegian and validated^[46]. HADS is a 14-item questionnaire designed to screen for depression (7 items) and anxiety (7 items). Each item is scored from 0 to 3, and consequently the total score ranges from 0 to 21 for either depression or anxiety^[46]. A higher score indicates an increased level of symptoms. In this study, depressive mood was defined as a HADS-D subscore ≥ 8 , which is considered to be the most relevant score for the screening of depressive symptoms in chronic disease^[47].

Sleep disturbance was measured with the first dimension of the Basic Nordic Sleep Questionnaire (BNSQ) on a 5-point Likert scale and dichotomized into normal sleep (scores 4 to 5) or sleep disturbance (scores 1 to 3)^[48].

Statistical analysis

Normally distributed continuous variables were described with means and standard deviation (SD), and variables with skewed distributions were described with medians

and ranges. Crude differences between pairs of continuous variables were analyzed using Student's *t*-test when normally distributed, otherwise a non-parametric test (Kruskal Wallis test) was used. The crude association between pairs of categorical variables was analyzed with Chi-square test. To explore possible associations between the selected variables and fatigue, multiple regression models were fitted using fatigue as the dependent variable. For chronic fatigue, a logistic regression was fitted, and for total fatigue scores, a linear regression was used. Age and gender plus the variables that were statistically significant ($P < 0.1$) in univariate analyses were entered into the final multiple models. P -values < 0.05 were considered statistically significant in the multivariate analyses. As our analyses were considered as exploratory, no corrections for multiple testing were applied. All tests were two-sided. IBM SPSS Statistics for Windows version 24.0 (IBM Corp. Armonk, NY, United States, Released 2016) was used for all statistical analyses.

Ethical considerations

The Regional Committee for Medical and Health Research Ethics approved the study (2012/845/REK). All the study participants gave written, informed consent before inclusion in the study, and the study was performed in accordance with the Declaration of Helsinki.

RESULTS

In total, 452 patients were eligible, and their participation was requested; of these, 414 (92%) gave written consent. Nine of these 414 patients were excluded due to missing data, leaving 405 patients available for the analyses, of which 227 (56%) had CD and 178 (44%) had UC. There were no statistically significant differences between the UC and CD patients regarding age or gender, but the CD patients had significantly longer disease duration than the UC patients (median 11 vs 6 years, $P < 0.01$). Approximately half (203/405) of the patients had vitamin D deficiency^[28]. For further details see Table 1.

The median score for total fatigue was similar in patients with UC and those with CD, see Table 1. There were no significant associations between vitamin D deficiency and mental, physical, and total fatigue scores. In addition, no significant differences in mean vitamin D levels between patients with and patients without chronic fatigue were found. Chronic fatigue was reported by 116 (29%) of all included patients with substantial fatigue (dichotomized FQ scores ≥ 4) reported by 194 (48%).

When separating patients into four groups according to (1) only chronic fatigue, (2) only depressive symptoms, (3) both chronic fatigue and depressive symptoms or (4) no chronic fatigue or depressive symptoms, there was no statistically significant association between the groups and their vitamin D levels, including when the data were adjusted for gender. No differences in total fatigue scores

or in the number of chronic fatigue cases were reported between patients receiving different medical treatments (data not shown).

In the multivariate linear regression analysis, when adjusted for age, gender, depressive symptoms, sleep disturbance and vitamin D level, higher total fatigue scores were associated with elevated disease activity scores in comparison to the total fatigue scores of patients in clinical remission in both UC and CD, but not with increased CRP or fecal calprotectin. In UC patients, female gender was associated with higher total fatigue scores in the univariate analysis, but this association disappeared in the multiple regression. Sleep disturbance and more depressive symptoms were associated with higher total fatigue in patients with UC and those with CD. The analyses are summarized in Tables 2 and 3.

In the multivariate logistic regression analysis, when adjusted for age, gender, depressive symptoms, sleep disturbance and vitamin D level, chronic fatigue was associated with elevated disease activity scores but not with increased CRP or fecal calprotectin, in contrast to patients in clinical remission regardless of diagnosis. In UC patients, female gender was associated with higher odds for chronic fatigue in the univariate analysis, but no such difference was found when adjusted for elevated disease activity scores. Self-reported sleep disturbance and depressive mood (HADS-D ≥ 8) were associated with higher odds for chronic fatigue in CD patients but not in UC patients in the multivariate analyses adjusted for disease activity and gender. The analyses are summarized in Tables 4 and 5.

DISCUSSION

In this cross-sectional study of IBD patients, neither total fatigue scores nor chronic fatigue were associated with vitamin D deficiency. Both higher total fatigue scores and chronic fatigue were associated with elevated disease activity scores, indicating clinically active disease in both UC and CD patients, but not with objective inflammatory markers. Moreover, higher total fatigue scores were associated with more depressive symptoms, as well as disturbed sleep in both disease groups.

Vitamin D deficiency was reported by half of the patients in this study, and as reported in a previous paper, this is more common than in the general population^[28]. Furthermore, vitamin D deficiency was associated with higher disease activity scores and relapse rates in CD, as well as increased inflammatory markers in UC^[28]. The prevalence of fatigue and mean total fatigue scores were similar to those reported in previous studies in IBD patients^[5,7,10].

To the best of our knowledge, no previous studies have investigated if vitamin D deficiency is associated with fatigue in IBD patients. However, vitamin D deficiency has been linked to fatigue in cancer patients^[21].

Table 1 Socio-demographic and clinical data *n* (%)

	UC (<i>n</i> = 178)	CD (<i>n</i> = 227)	<i>P</i> value
Age median, years (range)	40 (18-76)	40 (18-77)	0.92
Gender			
Female	87 (48)	112 (49)	0.77
Disease duration median, years (range)	6 (0-46)	11 (0-50)	< 0.01
Total fatigue, median (range)	16 (3-30)	15 (0-33)	0.55
Chronic fatigue	52 (29)	65 (28)	0.93
25-OH-D concentration nmol/L			
< 50	78 (44)	125 (55)	0.07
50-75	76 (43)	68 (30)	< 0.01
> 75	24 (13)	34 (15)	0.61
HADS-D ≥ 8	25 (14)	32 (14)	0.99
Sleep disturbance	44 (25)	36 (16)	0.03
Disease activity			
SCCAI ≥ 5	53 (29)		
HBI ≥ 5		103 (46)	
Fecal calprotectin ≥ 100 mg/kg (missing <i>n</i> = 82)	61 (34)	86 (38)	
CRP ≥ 5 mg/L (missing <i>n</i> = 10)	50 (28)	76 (33)	
UC extent, Montreal classification			
E1 - Proctitis	19 (11)		
E2 - Left-sided colitis	58 (32)		
E3 - Extensive colitis	101 (56)		
CD localization, Montreal classification			
L1 - Terminal ileum		74 (32)	
L2 - Colon		48 (21)	
L3 - Ileocolon		75 (33)	
L4 - Upper GI		31 (14)	
Current use of medication			
Biologics	52 (29)	113 (50)	< 0.01
5-ASA	137 (77)	23 (10)	< 0.01
Prednisolone	26 (14)	19 (8)	0.05
AZA / MTX	46 (26)	87 (38)	0.02

UC: Ulcerative colitis; CD: Crohn's disease; SCCAI: Simple Clinical Colitis Activity Index; HBI: Harvey Bradshaw index; ASA: Aminosalicic acid; AZA: Azathioprine; MTX: Methotrexate; CRP: C-reactive protein; HADS-D: Hospital Anxiety and depression score - depression subscore.

Moreover, supplementation of vitamin D decreased fatigue scores in a Swedish study in patients with neurological disease^[23]. In a study from the US of patients with different chronic conditions, not including IBD, vitamin D supplementation was also significantly associated with the improvement of fatigue symptoms^[22]. These findings may suggest a possible role of vitamin D deficiency in the development of fatigue. As previously discussed, one explanation behind such an effect may be that insufficient vitamin D levels predispose patients to muscular weakness and physical fatigue. In the current study, however, no association between physical fatigue and vitamin D deficiency was found.

In addition, vitamin D has immunoregulatory properties, which may attenuate intestinal inflammation^[30,33,49]. As fatigue is more often reported in patients with active disease, one could speculate that vitamin D deficiency may contribute to disease activity, which in turn increases fatigue^[5,10,16]. In the current study, however, vitamin D deficiency was not independently associated with fatigue. However, there was an association between fatigue and elevated disease activity scores in patients with UC and those with CD, but not with objective inflammatory markers such as CRP and fecal calprotectin. This suggests that factors other than inflammation may play an important role and that the total burden from

clinical disease activity may contribute relatively more to the experience of fatigue by the patient than just the intestinal inflammation.

Previous studies have also found pain and disturbed sleep to be of importance for the perception of fatigue in IBD^[17,50]. This is in accordance with our results, where symptoms of disturbed sleep were consistently associated with both total fatigue scores and chronic fatigue for both UC and CD patients. Sleep disturbance has been shown to be highly prevalent in inflammatory conditions including IBD and has also been associated with higher fatigue scores^[17,50]. Furthermore, there is evidence that sleep deprivation may increase inflammatory activity, but this has not been studied in IBD patients^[50].

The finding that several symptoms not directly related to intestinal inflammation may influence fatigue is supported by a Swedish study on fatigue in gastrointestinal disorders that reported more severe fatigue in patients with functional gastrointestinal disorders than in those with organic gastrointestinal disorders^[18]. Another Swedish study in irritable bowel syndrome has shown that patients with more severe symptoms report higher fatigue severity, supporting the importance of symptom burden in fatigue^[51].

In contrast to previous studies, however, we found that clinical disease activity, depressive symptoms and

Table 2 Univariate and multivariate analysis of total fatigue and selected variables in ulcerative colitis (*n* = 178)

	Univariate B	95%CI	P value	Multivariate B	95%CI	P value
Age	-0.07	-0.13, -0.01	0.03	-0.05	-0.10, 0.00	0.04
Sex (ref. male)	2.30	0.80, 3.80	< 0.01	1.29	0.01, 2.57	0.05
25-OH-D < 50 nmol/L	Ref.					
25-OH-D 50-75	-0.27	-1.92, 1.39	0.75			
25-OH-D > 75	-0.93	-3.33, 1.47	0.45			
SCCAI ≥ 5	5.66	4.20, 7.12	< 0.01	4.25	2.75, 5.75	< 0.01
CRP ≥ 5 mg/L	0.35	-1.37, 2.07	0.69			
Calprotectin ≥ 100 mg/kg	0.61	-1.18, 2.40	0.50			
Depressive symptoms ¹	4.78	2.68, 6.88	< 0.01	2.37	0.44, 4.30	0.02
Sleep disturbance	4.17	2.50, 5.84	< 0.01	2.07	0.47, 3.67	0.01

¹HADS: Hospital Anxiety and depression score - depression subscore ≥ 8. UC: Ulcerative colitis; SCCAI: Simple Clinical Colitis Activity Index; CRP: C-reactive protein; B: Regression coefficient.

Table 3 Univariate and multivariate analysis of total fatigue and selected variables in Crohn's disease (*n* = 227)

	Univariate B	95%CI	P value	Multivariate B	95%CI	P value
Age	-0.01	-0.07, 0.05	0.75	0.00	-0.06, 0.05	0.84
Sex (ref. male)	1.30	-0.16, 2.75	0.08	0.63	-0.67, 1.93	0.34
25-OH-D < 50 nmol/L	Ref.					
25-OH-D 50-75	-1.04	-2.79, 0.63	0.22			
25-OH-D > 75	-0.66	-2.79, 1.48	0.55			
HBI ≥ 5	4.17	2.81, 5.54	< 0.01	3.26	1.92, 4.59	< 0.01
CRP ≥ 5 mg/L	0.68	-0.89, 2.25	0.40			
Calprotectin ≥ 100 mg/kg	0.00	1.61, 1.61	1.00			
Depressive symptoms ¹	5.99	4.02, 7.94	< 0.01	4.72	2.82, 6.63	< 0.01
Sleep disturbance	4.04	2.10, 5.99	< 0.01	2.21	0.40, 4.03	0.02

¹HADS-D: Hospital Anxiety and depression score - depression subscore ≥ 8. CD: Crohn's disease; SCCAI: Simple Clinical Colitis Activity Index; CRP: C-reactive protein; B: Regression coefficient.

sleep disturbance are independently associated with fatigue. Depressive symptoms have consistently been associated with fatigue, mainly due to an overlap of symptoms between these conditions. We believe that these are separate conditions, even if fatigue has been shown to predict the onset of depression^[52]. Similar to our study, fatigue is often seen in patients with no history or current signs of psychological comorbidity^[53]. In a Canadian study, perceived psychological stress was associated with the presence of fatigue, but depressive symptoms were not measured^[17].

Previous studies in IBD patients have shown that fatigue is more commonly reported in female patients^[6,7]. A similar observation has been made in a Norwegian study on fatigue in the general population^[8]. In other studies, however, gender has not been shown to have a significant impact on fatigue^[10,19]. The latter is in accordance with the current study, where female gender was not associated with fatigue when adjusted for disease activity.

Even with a cross-sectional design of our study, the number of patients included is relatively high, and the patients were recruited from several hospitals. The patients included can therefore be assumed to be representative of IBD patients treated in specialist care settings. The sample size and completeness of data, with few missing data from questionnaires, are important

strengths in this study. The choice of questionnaire used to measure fatigue may be of relevance when comparing results from different studies as this may influence both the number of cases and severity reported. We used the Fatigue Questionnaire because it has been validated in the general Norwegian population^[8].

The duration of disease was longer in CD patients, and this may influence patient-reported outcomes such as fatigue, depressive symptoms and sleep disturbance. It is not known how disease duration influences these outcomes in chronic disease, but it may result in under-reporting, as patients may get used to fatigue symptoms over time. With self-reporting of symptoms, there is also a risk of recall bias.

Approximately 40% of the patients in the current study were treated with biologics. This may represent a selection bias of patients with more severe disease, with an expected higher prevalence of fatigue. On the other hand, effective medical treatment reducing the symptom burden may have improved fatigue scores in several patients. Only a few patients were treated on corticosteroids, not allowing for analyses on the effect use of steroids has on fatigue.

In conclusion, fatigue is common in IBD and is associated with clinical disease activity, depression and sleep disturbance. Our data did not reveal any association between vitamin D deficiency and fatigue, supporting

Table 4 Univariate and multivariate analysis of chronic fatigue in ulcerative colitis (*n* = 178)

	Univariate OR (95%CI)		P value	Multivariate OR (95%CI)		P value
Age, years	0.59	0.07, 1.02	0.59			
Gender (ref. male)	2.12	1.10, 4.11	0.025	1.80	0.90, 3.62	0.10
25-OH-D < 50 nmol/L (ref.)	1.00					
25-OH-D 50-75	0.93	0.45, 1.84	0.80			
25-OH-D > 75	1.20	0.45, 3.18	0.72			
SCCAI ≥ 5	4.81	2.39, 9.67	< 0.01	4.47	2.20, 9.07	< 0.01
CRP ≥ 5 mg/L	1.20	0.57, 2.52	0.63			
Calprotectin ≥ 100 mg/kg	1.57	0.73, 3.39	0.25			
Depressive symptoms ¹	3.92	1.64, 9.36	< 0.01	2.55	0.98, 6.64	0.06
Sleep disturbance	2.71	1.33, 5.53	< 0.01	1.40	0.61, 3.19	0.43

¹HADS-D: Hospital Anxiety and depression score - depression subscore ≥ 8. UC: Ulcerative colitis; SCCAI: Simple Clinical Colitis Activity Index; CRP: C-reactive protein.

Table 5 Univariate and multivariate analysis of chronic fatigue in Crohn's disease (*n* = 227)

	Univariate OR (95%CI)		P value	Multivariate OR (95%CI)		P value
Age, years	1.02	1.00, 1.04	0.10			
Gender (ref. male)	1.27	0.71, 2.26	0.41	1.11	0.61, 2.01	0.74
25-OH-D < 50 nmol/L (ref.)	1.00					
25-OH-D 50-75	0.65	0.33, 1.29	0.22			
25-OH-D > 75	1.25	0.56, 2.78				
HBI ≥ 5	2.71	1.50, 4.91	< 0.01	2.67	1.47, 4.87	< 0.01
CRP ≥ 5 mg/L	1.18	0.64, 2.19	0.59			
Calprotectin ≥ 100 mg/kg	0.56	0.29, 1.10	0.56			
Depressive symptoms ¹	4.77	2.19, 10.39	< 0.01	3.65	1.61, 8.27	< 0.01
Sleep disturbance	4.18	2.00, 8.74	< 0.01	3.02	1.38, 6.61	< 0.01

¹HADS-D: Hospital Anxiety and depression score - depression subscore ≥ 8. CD: Crohn's disease; HBI: Harvey Bradshaw index; CRP: C-reactive protein.

a multidimensional approach in the understanding of fatigue. The possible associations we report need to be explored in further clinical studies before any certain conclusions can be drawn.

ARTICLE HIGHLIGHTS

Research background

Fatigue is common in inflammatory bowel diseases (IBD) and is especially prevalent in active disease. Also sleep disturbance, anemia, pain and depression all seem to influence fatigue. However, a relationship between vitamin D and fatigue has not been established.

Research motivation

We wanted to investigate if vitamin D deficiency was associated to fatigue in IBD as this is a common belief among both patients and physicians. To the best of our knowledge, no previous studies have investigated this possible association in IBD patients.

Research objectives

A relationship between vitamin D and fatigue has not been established. We wanted to explore this association and discover possible implications for our patients and further research.

Research methods

The research question was explored in a fairly large cohort of IBD patients from specialist care. The study was designed as an observational study, and all data were collected at inclusion. Linear and logistic regression models were applied to explore the possible association between vitamin D deficiency and total fatigue scores and chronic fatigue, respectively. All vitamin D analyses were done at the same laboratory.

Research results

In this study fatigue was commonly reported. Vitamin D levels were, however, neither associated with total fatigue nor with chronic fatigue. Higher total fatigue scores and chronic fatigue were both associated with increased disease activity scores, but not with objective markers of inflammation. Sleep disturbance and depressive symptoms were associated with total fatigue scores in both ulcerative colitis and Crohn's disease (CD) patients, but with chronic fatigue only in CD patients.

Research conclusions

In this study, fatigue was associated with clinical disease activity, depression and sleep disturbance. Our data did not reveal any association between vitamin D deficiency and fatigue, supporting a multidimensional approach in the understanding of fatigue.

Research perspectives

The possible associations we report need to be explored in further clinical studies. A randomized controlled trial with an interventional group receiving vitamin D supplementation may shed light on the possible benefits of vitamin D in patient reported outcomes in IBD.

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REFERENCES

- 1 Ponder A, Long MD. A clinical review of recent findings in the epidemiology of inflammatory bowel disease. *Clin Epidemiol* 2013; 5: 237-247 [PMID: 23922506 DOI: 10.2147/CLEP.S33961]

- 2 **Cosnes J**, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1785-1794 [PMID: 21530745 DOI: 10.1053/j.gastro.2011.01.055]
- 3 **Ng SC**, Bernstein CN, Vatn MH, Lakatos PL, Loftus EV Jr, Tysk C, O'Morain C, Moum B, Colombel JF. Epidemiology and Natural History Task Force of the International Organization of Inflammatory Bowel Disease (IOIBD). Geographical variability and environmental risk factors in inflammatory bowel disease. *Gut* 2013; **62**: 630-649 [PMID: 23335431 DOI: 10.1136/gutjnl-2012-303661]
- 4 **Casellas F**, López-Vivancos J, Vergara M, Malagelada J. Impact of inflammatory bowel disease on health-related quality of life. *Dig Dis* 1999; **17**: 208-218 [PMID: 10754360 DOI: 10.1159/000016938]
- 5 **van Langenberg DR**, Gibson PR. Systematic review: fatigue in inflammatory bowel disease. *Aliment Pharmacol Ther* 2010; **32**: 131-143 [PMID: 20456309 DOI: 10.1111/j.1365-2036.2010.04347.x]
- 6 **Bager P**, Befrits R, Wikman O, Lindgren S, Moum B, Hjortswang H, Hjollund NH, Dahlerup JF. Fatigue in out-patients with inflammatory bowel disease is common and multifactorial. *Aliment Pharmacol Ther* 2012; **35**: 133-141 [PMID: 22059387 DOI: 10.1111/j.1365-2036.2011.04914.x]
- 7 **Huppertz-Hauss G**, Høivik ML, Jelsness-Jørgensen LP, Opheim R, Henriksen M, Høie O, Hovde Ø, Kempinski-Monstad I, Solberg IC, Jahnsen J, Hoff G, Moum B, Bernklev T. Fatigue in a population-based cohort of patients with inflammatory bowel disease 20 years after diagnosis: The IBSEN study. *Scand J Gastroenterol* 2017; **52**: 351-358 [PMID: 27852169 DOI: 10.1080/00365521.2016.1256425]
- 8 **Loge JH**, Ekeberg O, Kaasa S. Fatigue in the general Norwegian population: normative data and associations. *J Psychosom Res* 1998; **45**: 53-65 [PMID: 9720855]
- 9 **Smets EM**, Garssen B, Bonke B, De Haes JC. The Multidimensional Fatigue Inventory (MFI) psychometric qualities of an instrument to assess fatigue. *J Psychosom Res* 1995; **39**: 315-325 [PMID: 7636775]
- 10 **Jelsness-Jørgensen LP**, Bernklev T, Henriksen M, Torp R, Moum BA. Chronic fatigue is more prevalent in patients with inflammatory bowel disease than in healthy controls. *Inflamm Bowel Dis* 2011; **17**: 1564-1572 [PMID: 21674713 DOI: 10.1002/ibd.21530]
- 11 **Swain MG**. Fatigue in chronic disease. *Clin Sci (Lond)* 2000; **99**: 1-8 [PMID: 10887052]
- 12 **Moum B**, Vatn MH, Ekbohm A, Aadland E, Fausa O, Lygren I, Stray N, Sauar J, Schulz T. Incidence of Crohn's disease in four counties in southeastern Norway, 1990-93. A prospective population-based study. The Inflammatory Bowel South-Eastern Norway (IBSEN) Study Group of Gastroenterologists. *Scand J Gastroenterol* 1996; **31**: 355-361 [PMID: 8726303]
- 13 **Moum B**, Vatn MH, Ekbohm A, Aadland E, Fausa O, Lygren I, Sauar J, Schulz T, Stray N. Incidence of ulcerative colitis and indeterminate colitis in four counties of southeastern Norway, 1990-93. A prospective population-based study. The Inflammatory Bowel South-Eastern Norway (IBSEN) Study Group of Gastroenterologists. *Scand J Gastroenterol* 1996; **31**: 362-366 [PMID: 8726304]
- 14 **Loftus EV Jr**. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology* 2004; **126**: 1504-1517 [PMID: 15168363]
- 15 **Bernklev T**, Jahnsen J, Henriksen M, Lygren I, Aadland E, Sauar J, Schulz T, Stray N, Vatn M, Moum B. Relationship between sick leave, unemployment, disability, and health-related quality of life in patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2006; **12**: 402-412 [PMID: 16670530 DOI: 10.1097/01.MIB.0000218762.61217.4a]
- 16 **Czuber-Dochan W**, Ream E, Norton C. Review article: Description and management of fatigue in inflammatory bowel disease. *Aliment Pharmacol Ther* 2013; **37**: 505-516 [PMID: 23311461 DOI: 10.1111/apt.12205]
- 17 **Graff LA**, Vincent N, Walker JR, Clara I, Carr R, Ediger J, Miller N, Rogala L, Rawsthorne P, Lix L, Bernstein CN. A population-based study of fatigue and sleep difficulties in inflammatory bowel disease. *Inflamm Bowel Dis* 2011; **17**: 1882-1889 [PMID: 21830266 DOI: 10.1002/ibd.21580]
- 18 **Simrén M**, Svedlund J, Posserud I, Björnsson ES, Abrahamsson H. Predictors of subjective fatigue in chronic gastrointestinal disease. *Aliment Pharmacol Ther* 2008; **28**: 638-647 [PMID: 18564325 DOI: 10.1111/j.1365-2036.2008.03770.x]
- 19 **van Langenberg DR**, Gibson PR. Factors associated with physical and cognitive fatigue in patients with Crohn's disease: a cross-sectional and longitudinal study. *Inflamm Bowel Dis* 2014; **20**: 115-125 [PMID: 24297056 DOI: 10.1097/01.MIB.0000437614.91258.70]
- 20 **Graff LA**, Clara I, Walker JR, Lix L, Carr R, Miller N, Rogala L, Bernstein CN. Changes in fatigue over 2 years are associated with activity of inflammatory bowel disease and psychological factors. *Clin Gastroenterol Hepatol* 2013; **11**: 1140-1146 [PMID: 23602816 DOI: 10.1016/j.cgh.2013.03.031]
- 21 **Dev R**, Del Fabbro E, Schwartz GG, Hui D, Palla SL, Gutierrez N, Bruera E. Preliminary report: vitamin D deficiency in advanced cancer patients with symptoms of fatigue or anorexia. *Oncologist* 2011; **16**: 1637-1641 [PMID: 21964001 DOI: 10.1634/theoncologist.2011-0151]
- 22 **Roy S**, Sherman A, Monari-Sparks MJ, Schweiker O, Hunter K. Correction of Low Vitamin D Improves Fatigue: Effect of Correction of Low Vitamin D in Fatigue Study (EViDiF Study). *N Am J Med Sci* 2014; **6**: 396-402 [PMID: 25210673 DOI: 10.4103/1947-2714.139291]
- 23 **Askmark H**, Haggård L, Nygren I, Punga AR. Vitamin D deficiency in patients with myasthenia gravis and improvement of fatigue after supplementation of vitamin D3: a pilot study. *Eur J Neurol* 2012; **19**: 1554-1560 [PMID: 22672742 DOI: 10.1111/j.1468-1331.2012.03773.x]
- 24 **Ulitsky A**, Ananthakrishnan AN, Naik A, Skaros S, Zadornova Y, Binion DG, Issa M. Vitamin D deficiency in patients with inflammatory bowel disease: association with disease activity and quality of life. *JPEN J Parenter Enteral Nutr* 2011; **35**: 308-316 [PMID: 21527593 DOI: 10.1177/0148607110381267]
- 25 **Siffledeen JS**, Siminoski K, Steinhart H, Greenberg G, Fedorak RN. The frequency of vitamin D deficiency in adults with Crohn's disease. *Can J Gastroenterol* 2003; **17**: 473-478 [PMID: 12945007]
- 26 **Silvennoinen J**. Relationships between vitamin D, parathyroid hormone and bone mineral density in inflammatory bowel disease. *J Intern Med* 1996; **239**: 131-137 [PMID: 8568480]
- 27 **Sadeghian M**, Saneei P, Siassi F, Esmailzadeh A. Vitamin D status in relation to Crohn's disease: Meta-analysis of observational studies. *Nutrition* 2016; **32**: 505-514 [PMID: 26837598 DOI: 10.1016/j.nut.2015.11.008]
- 28 **Frigstad SO**, Høivik M, Jahnsen J, Dahl SR, Cvancarova M, Grimstad T, Berset IP, Huppertz-Hauss G, Hovde Ø, Torp R, Bernklev T, Moum B, Jelsness-Jørgensen LP. Vitamin D deficiency in inflammatory bowel disease: prevalence and predictors in a Norwegian outpatient population. *Scand J Gastroenterol* 2017; **52**: 100-106 [PMID: 27603182 DOI: 10.1080/00365521.2016.1233577]
- 29 **Jahnsen J**, Falch JA, Mowinkel P, Aadland E. Vitamin D status, parathyroid hormone and bone mineral density in patients with inflammatory bowel disease. *Scand J Gastroenterol* 2002; **37**: 192-199 [PMID: 11843057]
- 30 **Jørgensen SP**, Hvas CL, Agnholt J, Christensen LA, Heickendorff L, Dahlerup JF. Active Crohn's disease is associated with low vitamin D levels. *J Crohns Colitis* 2013; **7**: e407-e413 [PMID: 23403039 DOI: 10.1016/j.crohns.2013.01.012]
- 31 **Blanck S**, Abera F. Vitamin d deficiency is associated with ulcerative colitis disease activity. *Dig Dis Sci* 2013; **58**: 1698-1702 [PMID: 23334382 DOI: 10.1007/s10620-012-2531-7]
- 32 **Kabbani TA**, Koutroubakis IE, Schoen RE, Ramos-Rivers C, Shah N, Swoger J, Regueiro M, Barrie A, Schwartz M, Hashash JG, Baidoo L, Dunn MA, Binion DG. Association of Vitamin D Level With Clinical Status in Inflammatory Bowel Disease: A 5-Year Longitudinal Study. *Am J Gastroenterol* 2016; **111**: 712-719 [PMID: 26952579 DOI: 10.1038/ajg.2016.53]
- 33 **Holick MF**. Vitamin D deficiency. *N Engl J Med* 2007; **357**: 266-281 [PMID: 17634462 DOI: 10.1056/NEJMra070553]
- 34 **Lennard-Jones JE**. Classification of inflammatory bowel disease.

- Scand J Gastroenterol Suppl* 1989; **170**: 2-6; discussion 16-9 [PMID: 2617184]
- 35 **Cashman KD**, Dowling KG, Škrabáková Z, Kiely M, Lamberg-Allardt C, Durazo-Arvizu RA, Semplos CT, Koskinen S, Lundqvist A, Sundvall J, Linneberg A, Thuesen B, Husemoen LL, Meyer HE, Holvik K, Grønberg IM, Tetens I, Andersen R. Standardizing serum 25-hydroxyvitamin D data from four Nordic population samples using the Vitamin D Standardization Program protocols: Shedding new light on vitamin D status in Nordic individuals. *Scand J Clin Lab Invest* 2015; **75**: 549-561 [PMID: 26305421 DOI: 10.3109/00365513.2015.1057898]
 - 36 **Travis SP**, Higgins PD, Orchard T, Van Der Woude CJ, Panaccione R, Bitton A, O'Morain C, Panés J, Sturm A, Reinisch W, Kamm MA, D'Haens G. Review article: defining remission in ulcerative colitis. *Aliment Pharmacol Ther* 2011; **34**: 113-124 [PMID: 21615435 DOI: 10.1111/j.1365-2036.2011.04701.x]
 - 37 **D'Haens G**, Sandborn WJ, Feagan BG, Geboes K, Hanauer SB, Irvine EJ, Lémann M, Marteau P, Rutgeerts P, Schölmerich J, Sutherland LR. A review of activity indices and efficacy end points for clinical trials of medical therapy in adults with ulcerative colitis. *Gastroenterology* 2007; **132**: 763-786 [PMID: 17258735 DOI: 10.1053/j.gastro.2006.12.038]
 - 38 **Kristensen V**, Klepp P, Cvancarova M, Røseth A, Skar V, Moum B. Prediction of endoscopic disease activity in ulcerative colitis by two different assays for fecal calprotectin. *J Crohns Colitis* 2015; **9**: 164-169 [PMID: 25518057 DOI: 10.1093/ecco-jcc/jju015]
 - 39 **Harvey RF**, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet* 1980; **1**: 514 [PMID: 6102236]
 - 40 **Best WR**. Predicting the Crohn's disease activity index from the Harvey-Bradshaw Index. *Inflamm Bowel Dis* 2006; **12**: 304-310 [PMID: 16633052 DOI: 10.1097/01.MIB.0000215091.77492.2a]
 - 41 **Walmsley RS**, Ayres RC, Pounder RE, Allan RN. A simple clinical colitis activity index. *Gut* 1998; **43**: 29-32 [PMID: 9771402]
 - 42 **Gasche C**, Berstad A, Befrits R, Beglinger C, Dignass A, Erichsen K, Gomollon F, Hjortswang H, Koutroubakis I, Kulnigg S, Oldenburg B, Rampton D, Schroeder O, Stein J, Travis S, Van Assche G. Guidelines on the diagnosis and management of iron deficiency and anemia in inflammatory bowel diseases. *Inflamm Bowel Dis* 2007; **13**: 1545-1553 [PMID: 17985376 DOI: 10.1002/ibd.20285]
 - 43 **Vermeire S**, Schreiber S, Sandborn WJ, Dubois C, Rutgeerts P. Correlation between the Crohn's disease activity and Harvey-Bradshaw indices in assessing Crohn's disease severity. *Clin Gastroenterol Hepatol* 2010; **8**: 357-363 [PMID: 20096379 DOI: 10.1016/j.cgh.2010.01.001]
 - 44 **Chalder T**, Berelowitz G, Pawlikowska T, Watts L, Wessely S, Wright D, Wallace EP. Development of a fatigue scale. *J Psychosom Res* 1993; **37**: 147-153 [PMID: 8463991]
 - 45 **Pawlikowska T**, Chalder T, Hirsch SR, Wallace P, Wright DJ, Wessely SC. Population based study of fatigue and psychological distress. *BMJ* 1994; **308**: 763-766 [PMID: 7908238]
 - 46 **Zigmond AS**, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand* 1983; **67**: 361-370 [PMID: 6880820]
 - 47 **Bjelland I**, Dahl AA, Haug TT, Neckelmann D. The validity of the Hospital Anxiety and Depression Scale. An updated literature review. *J Psychosom Res* 2002; **52**: 69-77 [PMID: 11832252]
 - 48 **Partinen M**, Gislason T. Basic Nordic Sleep Questionnaire (BNSQ): a quantitated measure of subjective sleep complaints. *J Sleep Res* 1995; **4**: 150-155 [PMID: 10607192]
 - 49 **Shivananda S**, Lennard-Jones J, Logan R, Fear N, Price A, Carpenter L, van Blankenstein M. Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). *Gut* 1996; **39**: 690-697 [PMID: 9014768]
 - 50 **Ranjbaran Z**, Keefer L, Farhadi A, Stepanski E, Sedghi S, Keshavarzian A. Impact of sleep disturbances in inflammatory bowel disease. *J Gastroenterol Hepatol* 2007; **22**: 1748-1753 [PMID: 17914945 DOI: 10.1111/j.1440-1746.2006.04820.x]
 - 51 **Frändemark Å**, Jakobsson Ung E, Törnblom H, Simrén M, Jakobsson S. Fatigue: a distressing symptom for patients with irritable bowel syndrome. *Neurogastroenterol Motil* 2017; **29** [PMID: 27401139 DOI: 10.1111/nmo.12898]
 - 52 **Dryman A**, Eaton WW. Affective symptoms associated with the onset of major depression in the community: findings from the US National Institute of Mental Health Epidemiologic Catchment Area Program. *Acta Psychiatr Scand* 1991; **84**: 1-5 [PMID: 1927557]
 - 53 **van der Linden G**, Chalder T, Hickie I, Koschera A, Sham P, Wessely S. Fatigue and psychiatric disorder: different or the same? *Psychol Med* 1999; **29**: 863-868 [PMID: 10473313]

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Fourth-generation quinolones in the treatment of *Helicobacter pylori* infection: A meta-analysis

Ying An, Ya Wang, Shuang Wu, You-Hua Wang, Xing Qian, Zhen Li, Ying-Jun Fu, Yong Xie

Ying An, Ya Wang, Shuang Wu, You-Hua Wang, Xing Qian, Yong Xie, Department of Gastroenterology, the First Affiliated Hospital of Nanchang University, Key Laboratory of Digestive Diseases of Jiangxi, Nanchang 330000, Jiangxi province, China

Ying An, Ya Wang, Ying-Jun Fu, School of Pharmacy, Nanchang University, Nanchang 330000, Jiangxi province, China

Zhen Li, Medical College, Nanchang University, Nanchang 330000, Jiangxi province, China

ORCID number: Ying An (0000-0003-2332-3825); Ya Wang (0000-0001-6644-2993); Shuang Wu (0000-0002-2849-6437); You-Hua Wang (0000-0002-0157-0721); Xing Qian (0000-0002-5209-0584); Zhen Li (0000-0002-2227-7612); Ying-Jun Fu (0000-0001-8884-7860); Yong Xie (0000-0002-5290-5579).

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Correspondence to: Yong Xie, MD, PhD, Professor, Department of Gastroenterology, the First Affiliated Hospital of Nanchang University, Key Laboratory of Digestive Diseases of Jiangxi, Nanchang 330000, Jiangxi Province, China. xieyong_tfahoncu@163.com
Telephone: +86-791-88692507

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Abstract

AIM

To assess the efficacy and safety of fourth-generation quinolones for *Helicobacter pylori* (*H. pylori*) eradication, we conducted this systematic review and meta-analysis of randomized clinical trials.

METHODS

Major literature databases (PubMed, EMBASE and the Cochrane Central Register of Controlled Trials) were searched for relevant articles published prior to February 2018. We performed a meta-analysis of all randomized clinical trials that examined the efficacy of *H. pylori* eradication therapies and included fourth-generation quinolones in the experimental arm. Subgroup analyses by regions and different types of fourth-generation quinolones were also performed.

RESULTS

Ten studies including a total of 2198 patients were assessed. A meta-analysis of randomized controlled trials showed that the eradication rate of therapies containing non-fourth-generation quinolones was significantly lower

than that of therapies containing fourth-generation quinolones by intention-to-treat (ITT) analysis [75.4% *vs* 81.8%; odds ratio (OR) = 0.661; 95% confidence interval (CI): 0.447-0.977; *P* = 0.038]. This analysis also showed that the eradication rate of the therapies containing non-fourth-generation quinolones was inferior to that of therapies containing fourth-generation quinolones by per-protocol analysis (79.1% *vs* 84.7%; OR = 0.663; 95%CI: 0.433-1.016; *P* = 0.059). Moreover, the occurrence of side effects was significantly different between the control and experimental groups by ITT analysis (30.6% *vs* 19.5%; OR = 1.874; 95%CI: 1.120-3.137; *P* = 0.017). The sub-analyses also showed significant differences in moxifloxacin therapies *vs* other fourth-generation quinolone therapies (84.3% *vs* 71.9%) and in Asian *vs* European groups (76.7% *vs* 89.1%).

CONCLUSION

Therapies containing fourth-generation quinolones achieved a poor eradication rate in the treatment of *H. pylori* infection. Such regimens might be useful as a rescue treatment based on antimicrobial susceptibility testing. Different antibiotics should be chosen in different regions.

Key words: *Helicobacter pylori*; Fourth-generation quinolones; Eradication; Systematic review; Meta-analysis

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Core tip: With the increase in the *Helicobacter pylori* (*H. pylori*) resistance rate, eradication is becoming increasingly challenging. This is the first meta-analysis comprehensively focused on fourth-generation quinolones for the treatment of *H. pylori* infection. Additionally, we found that fourth-generation quinolones had a higher eradication rate (81.8%) and a lower rate of incidence of side effects (19.5%). These findings will provide a specific basis for the clinical use of fourth-generation quinolones for *H. pylori* eradication.

An Y, Wang Y, Wu S, Wang YH, Qian X, Li Z, Fu YJ, Xie Y. Fourth-generation quinolones in the treatment of *Helicobacter pylori* infection: A meta-analysis. *World J Gastroenterol* 2018; 24(29): 3302-3312 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i29/3302.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i29.3302>

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection plays a crucial role in the pathogenesis of gastrointestinal diseases, such as gastritis, non-ulcer dyspepsia, peptic ulcer diseases, and gastric cancer^[1]. *H. pylori* infection affects approximately 50% of the population worldwide^[2]. Its prevalence is approximately 70% in developing nations and approximately 20%-30% in developed nations^[3]. Eradication of *H. pylori* facilitates peptic ulcer healing, reduces ulcer relapse rates, and prevents gastric

cancer^[4]. In the past, the recommended treatment for eradicating *H. pylori* was 7 d of standard triple therapy (STT) consisting of a proton pump inhibitor (PPI) with clarithromycin (CAM) and amoxicillin (AMPC)^[5]. However, with the wide use of the STT regimen, the eradication rate of *H. pylori* has declined to unacceptable levels over the last decade (< 80%) due to high resistance to metronidazole and clarithromycin^[6]. A recent study on *H. pylori* resistance to antimicrobial agents reported that clarithromycin resistance has rapidly increased in many countries over the past decade, with resistance rates of approximately 18% in Europe, 30% in Japan, 40% in Turkey, and 50% in China; limited data are available for the United States^[7-10]. The prevalence of *H. pylori* resistance to metronidazole is 33% in Europe and 40% in the United States, with a high resistance rate (50%-80%) in developing countries^[10]. To overcome these difficulties, there is a need to evaluate novel regimens and antibiotics to identify effective alternative treatment strategies. Levofloxacin-based therapy is recommended by the Maastricht IV^[11] and Maastricht V Consensus Reports^[12]. Nonetheless, according to studies, the resistance rate to levofloxacin is approximately 22.1% in Italy and 36.9% in China; a recent study surprisingly reported a resistance rate of 31.9% in the United States^[13-15]. Jeong *et al*^[16] reported that the eradication rate was 57.1% when levofloxacin was used. Fourth-generation quinolones, including moxifloxacin, sitafloxacin, gemifloxacin, and gatifloxacin, which have broad-spectrum antibacterial activity, are active against a variety of gram-negative and gram-positive bacteria^[17]. Recent studies have shown that fourth-generation quinolones can increase drug penetration into bacterial cells, improve the strength of activity and have better bioavailability. This group of drugs inhibits the metabolism of bacterial cells by inhibiting DNA replication and therefore enhances antibacterial activity^[18]. Furthermore, treatment with fourth-generation quinolones has achieved a high *H. pylori* eradication rate and has been recommended in some studies^[19-22]. Nevertheless, Chung *et al*^[23] reported that the eradication rate was not satisfactory when using fourth-generation quinolones.

To evaluate the efficacy and safety of therapies containing fourth-generation quinolones, we conducted a systematic review and meta-analysis of the available data. The primary outcome measures we assessed were eradication rates, side effects, and compliance of the therapies containing fourth-generation quinolones compared with those of therapies containing non-fourth-generation quinolones. Our outcomes will provide useful evidence for clinical practice^[24].

MATERIALS AND METHODS

Literature sources

We searched the PubMed (to February 2018), EMBASE (to February 2018), and Cochrane Central Register of Controlled Trials (Issue 2, 2018) databases. The

following search terms were used for all databases: ("Helicobacter pylori" OR *H. pylori*) AND (Moxifloxacin OR Sitafloracin OR Gemifloxacin OR Gatifloxacin); the search terms varied slightly among these databases. This meta-analysis was conducted according to the Preferred Reporting Item for Systematic Reviews and Meta-Analyses (PRISMA)^[25].

Inclusion criteria

Articles eligible for inclusion in the meta-analysis met the following criteria: (1) randomized controlled trial (RCT) conducted; (2) methods used for the diagnosis of *H. pylori*, including the urea breath test (UBT), the rapid urease test (RUT), bacterial culture, histology, and/or fecal antigen test; (3) eradication rate made available; (4) eradication testing with UBT and/or histology performed at least 4 wk after the completion of therapy; and (5) eradication regimens in the experimental arm included fourth-generation quinolones.

Exclusion criteria

Studies were excluded under the following circumstances: (1) eradication data could not be confirmed; (2) articles and abstracts were written in a language other than English; (3) fourth-generation quinolones were included in two treatment arms; and (4) the experimental group and the control group included more than one variable (for example, the comparison of triple therapy and quadruple therapy; antibiotics and duration were different in both groups).

Data extraction

Three authors (An Y, Wang Y, and Wu S) independently extracted data from the selected studies. Any disagreements were resolved by consensus.

The extracted data included the following: the study design; number of enrolled patients in each treatment arm; diagnostic methods for confirming *H. pylori* infection before enrolling and re-checking strategies after completing the eradication study; publication time; name of the authors; location of the trial; drug regimens; duration of treatment; eradication rates by intention-to-treat (ITT) analysis and per-protocol (PP) analysis; number of successful and failed eradications; and percentage of adverse effects.

To avoid duplication of data, if a trial was repeatedly published by the same authors or institutions, only the most recently published or most informative study was included.

Risk of bias

The quality of RCTs with available full text was assessed using the risk of bias assessment tool developed by the Cochrane Handbook for Systematic Reviews of Interventions^[26]. Two independent reviewers assessed the risk of bias through six domain-based evaluations, including selection bias (random sequence generation and allocation concealment), performance bias (blinding

of participants and personnel), detection bias (blinding of outcome assessment), attrition bias (incomplete outcome data), reporting bias (selective outcome reporting), and other bias. Each indicator was scored by low risk of bias, unclear risk of bias, and high risk of bias. Any disagreement was discussed and decided by a third reviewer. We also employed a funnel plot and Egger's test to assess the presence of publication bias.

Statistical analysis

The statistical analysis was performed using the meta-analysis software STATA12.0 (StataCorp LP, College Station, TX, United States). The primary outcomes of the meta-analysis were the *H. pylori* eradication rate and therapy-related side effects among the trials comparing the control and experimental groups based on ITT analysis. For each trial, we calculated the odds ratio (OR) for the primary measure. The ORs were presented with 95% confidence intervals (CIs); in addition, a *P*-value < 0.05 was considered significant. The degree of heterogeneity among the trial results was estimated using the χ^2 statistic (*P*-value < 0.10 considered significant) and the *I*² test (0%-25%, 25%-50%, 50%-75%, and > 75% represented insignificant, low, moderate, and high heterogeneity, respectively). If significant heterogeneity (*P* < 0.10 or *I*² > 50%) was achieved, we employed the random effects model to combine the effect sizes of the included studies. When no significant heterogeneity was found, we used fixed effects to pool the data. Additionally, subgroup analyses were performed based on the location and different types of fourth-generation quinolones.

RESULTS

Description of the studies

The bibliographical search yielded a total of 548 studies from PubMed, Embase, and the Cochrane Central Register of Controlled Trials. Among these articles, we excluded 144 due to duplication and 175 that were unrelated. We selected 229 potential studies for detailed assessment, among which 68 were excluded because there was no control group. We also excluded 67 review articles, comments, or letters. Thirty-three articles were excluded because of the inclusion of fourth-generation quinolones in two regimens, 14 articles were non-RCTs, 11 articles were excluded due to an inappropriate drug regimen, and 19 articles had data that could not be determined. Then, 17 articles were selected for further evaluation. Five articles were excluded because the data repeated those in other studies, one study did not state the methods for diagnosis of *H. pylori*, and one study was published in Japanese. Ultimately, 10 studies (two abstracts and eight full-text articles) met the inclusion criteria and were included in the systematic review and meta-analysis (Figure 1). These 10 studies^[27-36] are summarized in Table 1 based on our meta-analysis. The quality assessment is reported in Table 2.

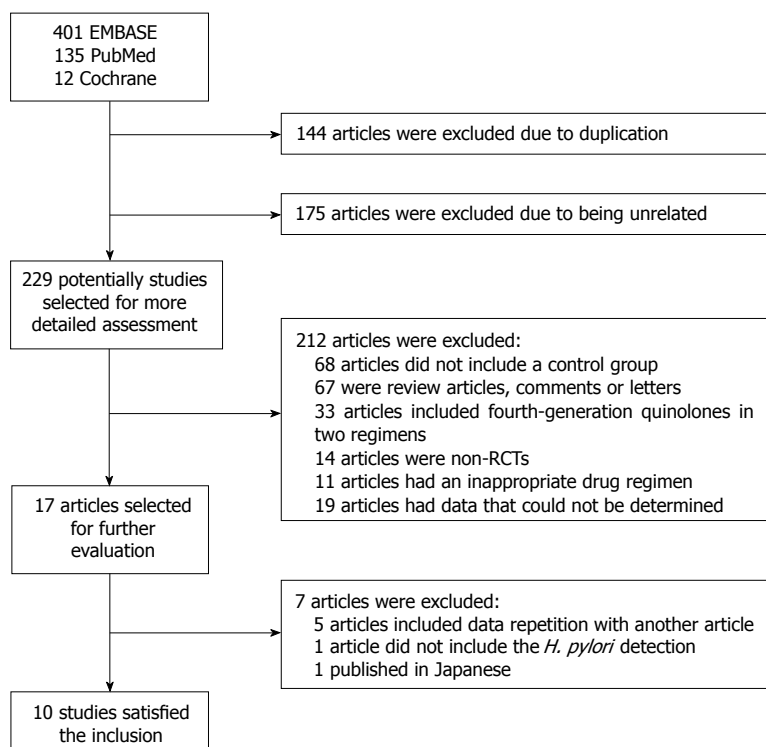


Figure 1 Flow diagram of the identified and selected trials.

Efficacy of *H. pylori* eradication

There were 10 studies with a total of 2198 patients in our meta-analysis; of these patients, 1107 received therapy without fourth-generation quinolone and 1091 received therapy with fourth-generation quinolone. The pooled eradication rates were 75.4% (835/1107) in the control group and 81.8% (892/1091) in the experimental group by ITT analysis. The pooled OR was 0.661 (95%CI: 0.447-0.977; $P = 0.038$) using the random effects model ($I^2 = 66.2\%$, $P = 0.000$; Figure 2).

Moreover, the pooled eradication rates were 79.1% (835/1055) in the control group and 84.7% (892/1053) in the experimental group by PP analysis. The pooled OR was 0.663 (95%CI: 0.433-1.016; $P = 0.059$) using the random effects model ($I^2 = 64.4\%$, $P = 0.000$).

The results of ITT showed that the eradication rates of therapies containing non-fourth-generation quinolones was significantly lower than those of therapies containing fourth-generation quinolones.

Subgroup analyses

Additional subgroup analyses for the meta-analysis were performed due to heterogeneity. We analyzed different types of fourth-generation quinolones, covering seven moxifloxacin trials and three other trials (including 1 sitafloxacin and 2 gemifloxacin). In the moxifloxacin subgroup, the pooled eradication rates were 77.3% (689/891) in the control group and 84.3% (733/870) in the experimental group (OR = 0.614, 95%CI: 0.403-0.935; $P = 0.023$; Figure 3) by ITT analysis, and the rates were 81.3% (689/847) in the control group and 87.3% (689/891) in the experimental group by

PP analysis (OR = 0.614, 95%CI: 0.395-0.956; $P = 0.031$). In the other subgroup, the pooled eradication rates were 67.6% (146/216) in the control group and 71.9% (159/221) in the experimental group (OR = 0.846, 95%CI: 0.274-2.614; $P = 0.772$; Figure 3) by ITT analysis, and the rates were 70.2% (146/208) in the control group and 74.6% (159/213) in the experimental group by PP analysis (OR = 0.860, 95%CI: 0.239-3.091; $P = 0.817$). This subgroup analysis showed that the regimen with moxifloxacin achieved a higher eradication rate than the regimen without moxifloxacin. However, there was no significant difference in the eradication rate in the other subgroup.

We also conducted subgroup analysis by region (seven trials in Asia and three trials in Europe). In the Asian subgroup, the pooled eradication rates of the control group and the experimental group were 76.5% (488/638) and 76.7% (493/643), respectively, by ITT analysis (OR = 1.051; 95%CI: 0.671-1.646; $P = 0.827$; Figure 4) and 79.6% (488/613) and 80.0% (493/616), respectively, by PP analysis (OR = 1.072; 95%CI: 0.627-1.833; $P = 0.800$). In the European subgroup, the pooled eradication rates of the control group and the experimental group were 74.0% (347/469) and 89.1% (399/448), respectively, by ITT analysis (OR = 0.661; 95%CI: 0.447-0.977; $P = 0.000$; Figure 4) and 78.5% (347/442) vs 91.3% (399/437), respectively, by PP analysis (OR = 0.361; 95% CI: 0.240-0.544; $P = 0.000$). The results showed that therapies containing fourth-generation quinolones may not be advisable treatments for *H. pylori* infection in Asia. However, the use of fourth-generation quinolones in Europe can

Table 1 Characteristics of studies included in the meta-analysis

Year-Author	Location	<i>H. pylori</i> infection diagnosis/re-checking	Control group -Day	Fourth-generation quinolone group-Day	Eradication rate (ITT) (control group/fourth-generation quinolone group)	Eradication rate (PP) (control group/fourth-generation quinolone group)	Compliance	Side effects
2017-Mansour Ghanaei, F	Iran	14C-UBT or histology	BPAC-10	BPAG-10	89% (81/91)/77% (70/91)	91% (81/90)/77.8% (70/90)		
2015-Masoodi, M	Iran	13C-UBT, RUT pathology test	OBAC-10	OBAG-10	61.6% (37/60)/66.6% (40/60)	67.2% (37/55)/72.7% (40/55)	97.1%/98.3%	37/19
2014-Rakici, H	Turkey	pistology, stool antigen test	LanAL-10	LanAM-10	89.4% (92/103)/87.8% (93/106)	92% (92/100)/91.8% (93/102)	96%/95.1%	-
2013-Murakami, K	Japan	culture method, RUT, UBT	LanAL-7	LanAS-7	43.1% (28/65)/70% (49/70)	43.7% (28/84)/72.1% (49/68)	98.4%/94.1%	11/11
2012-Zeng, Z	China	14C-UBT	EAC-7	EAM-7	78.9% (180/228)/79.4% (181/228)	82.9% (180/217)/84.2% (181/215)	-	-
2009-Lu, NH	China	14C-UBT	EAC-7	EAM-7	90.3% (28/31)/85.7% (24/28)	-	-	-
2008-Kilic, ZM	Turkey	gastroscopy, histology, RUT, 13C-UBT	RBCAC-14	RBCAM-14	76.7% (23/30)/66.7% (20/30)	76.7% (23/30)/66.7% (20/30)	100%/100%	11/13
2007-Bago, P	Croatia	RUT, histology, culture test, 13C-UBT	EAC-14	EAM-14	63.3% (19/30)/53.3% (16/30)	63.3% (19/30)/53.3% (16/30)	100%/100%	17/21
2005-Kist, M	Germany	RUT, histology, culture test, 13C-UBT	LanMetC-7	LanMetM-7	70.4% (50/71)/93.5% (58/62)	75.8% (50/66)/96.7% (58/60)	-	-
2005-Nista, EC	Italy	13C-UBT	LanAC-7	LanAM-7	78.2% (61/78)/86.4% (57/66)	80.2% (61/76)/90.5% (57/63)	-	-
		13C-UBT	EAC-7	EAM-7	72.5% (58/80)/87.5% (70/80)	78% (58/74)/89% (70/79)	-	-
		Histological examination, 13C-UBT	ETC-7	ETM-7	75% (60/80)/90% (72/80)	79% (60/76)/92.3% (72/78)	-	-
			ETC-7	ETM-7	75% (60/80)/90% (72/80)	78.9% (60/76)/92.3% (72/78)	-	29/11
			EAC-7	EAM-7	72.5% (58/80)/87.5% (70/80)	78.4% (58/74)/88.6% (70/79)	-	26/10

A: Amoxicillin; B: Bismuth; C: Clarithromycin; E: Esomeprazole G: Gemifloxacin; Lan: Lansoprazole; L: Levofloxacin; M: Moxifloxacin; Met: Metronidazole; O: Omeprazole; P: Pantoprazole; S: Sitafloxacin; RBC: Ranitidine bismuth citrate; -: Not reported.

significantly improve the eradication rate.

Side effects

Of the 10 studies, four studies provided data regarding side effects. The results showed that common symptoms included nausea, diarrhea, black stool, and taste disturbance. The occurrence of total side effects in the control group was significantly higher than that in the experimental group by ITT analysis (30.6% vs 19.5%, OR = 1.874; 95%CI: 1.120-3.137; $P = 0.017$; Figure 5).

Compliance

Four studies included in the meta-analysis provided information about compliance. The results showed high compliance (> 95%), and there were no significant differences between the study groups.

Risk of bias in publication

Egger's regression test suggested that there was no significant bias ($P = 0.725$) in the ITT analysis, while the funnel plot showed a slightly asymmetrical distribution (Figure 6).

DISCUSSION

H. pylori infection is marked by a vast prevalence and strong association with various gastric diseases^[37]. Therapeutic regimens range from STT to the present novel regimens,

Table 2 Risk assessment of included studies

Year-Author	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
2017-Mansour Ghanaei, F	L	H	H	L	L	L	L
2015-Masoodi, M	L	L	L	H	L	L	L
2014-Rakici, H	L	H	H	H	L	U	L
2013-Murakami, K	L	H	L	H	L	L	L
2012-Zeng, Z	U	U	U	U	L	U	U
2009-Lu, NH	U	U	U	U	L	U	U
2008-Kilic, ZM	L	H	H	H	L	L	U
2007-Bago, P	L	L	L	H	L	L	L
2005-Kist, M	H	H	H	H	L	H	L
2005- Nista, EC	L	H	H	H	L	L	L

L: Low risk of bias; H: High risk of bias; U: Unclear risk of bias.

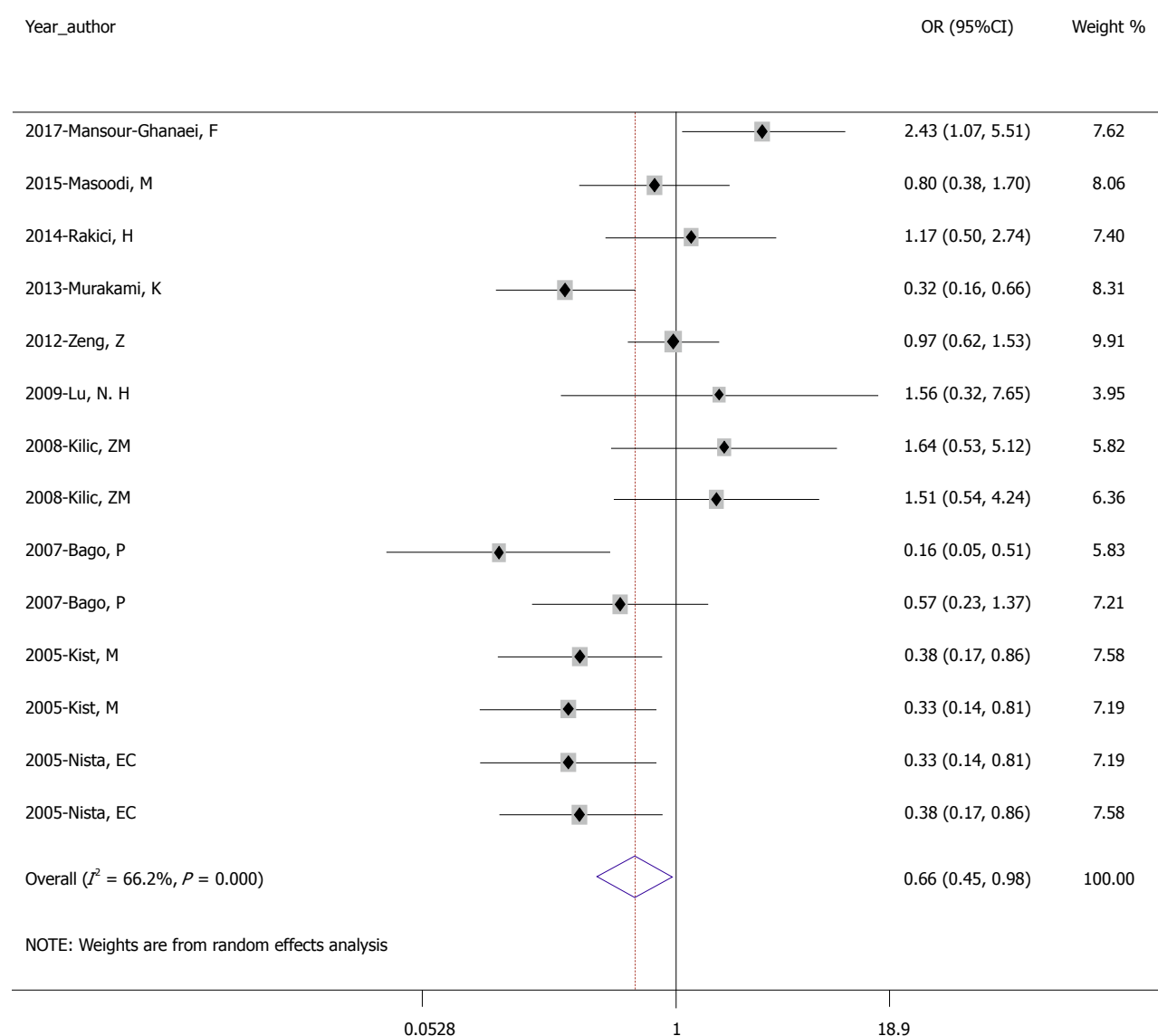


Figure 2 Forest plot of eradication rate of the therapies containing non-fourth-generation quinolones vs that of the therapies containing fourth-generation quinolones (intention-to-treat analysis).

such as quadruple therapy with bismuth, sequential treatment, concomitant therapy, and hybrid therapy^[38,39]. However, the treatment effects are still not ideal due to

bacterial antibiotic resistance^[40]. Thus, it is necessary to evaluate novel regimens or antibiotics^[41]. With the resistance rate to the third-generation quinolone

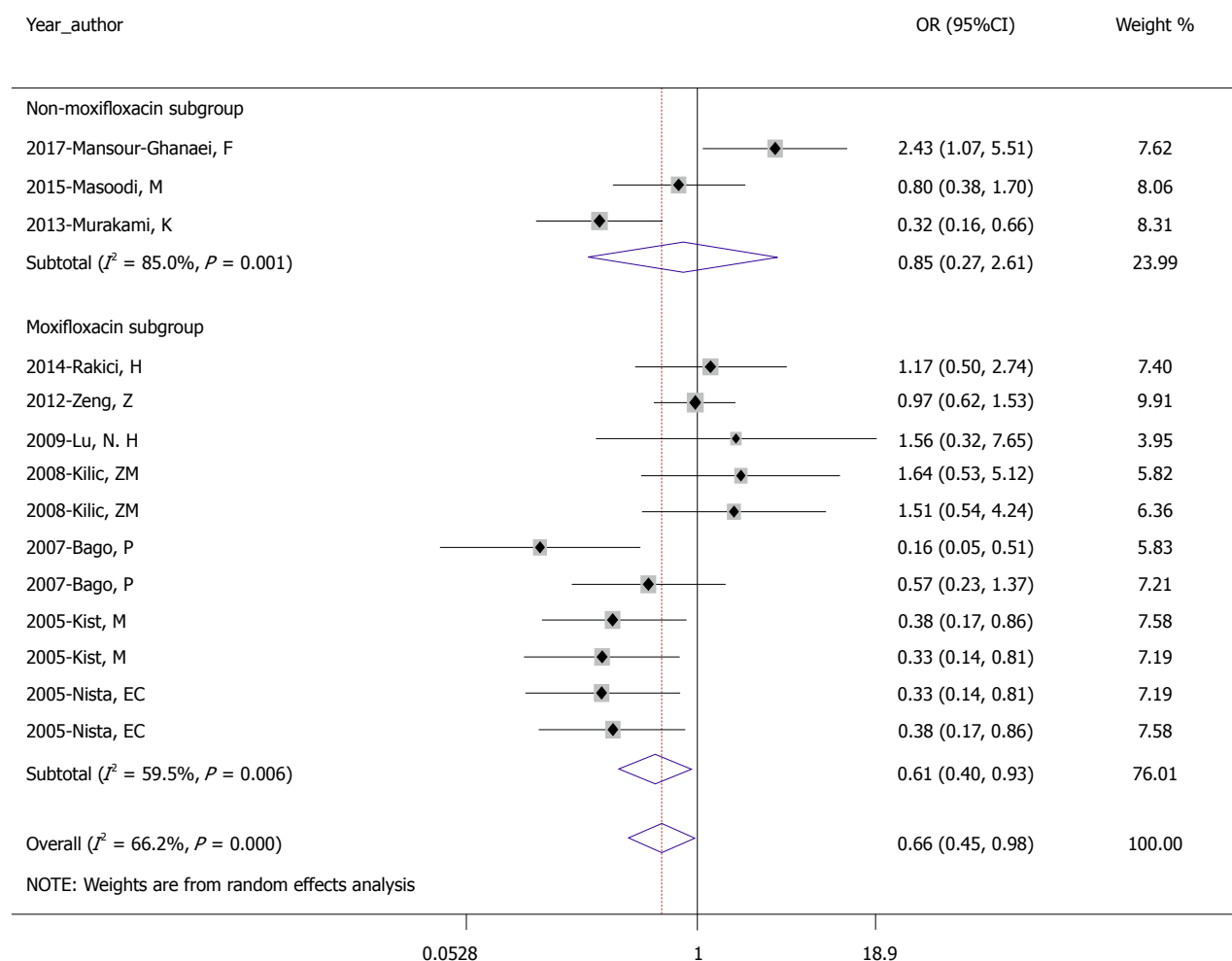


Figure 3 Forest plot of the sub-analysis according to types of fourth-generation quinolone (intention-to-treat analysis).

levofloxacin continuing to increase, resulting in a low eradication rate^[16], therapies containing fourth-generation quinolones might be suitable for the treatment of *H. pylori* infection.

This meta-analysis indicated that therapies containing fourth-generation quinolones had a higher clearance rate than other therapies by ITT and PP analyses. The mechanism of action of fourth-generation quinolones against *H. pylori* is to inhibit bacterial DNA gyrase, thus interfering with bacterial DNA replication^[42]. These fourth-generation quinolones embed in the broken DNA chain and form complexes to inhibit nicking and closing activity, achieving a bactericidal effect^[43]. However, according to Graham, who had given a report card to grade *H. pylori* therapy by ITT, the eradication rate is still poor (grade D, 81%-84%)^[44]. This may be related to the low compliance of patients^[27,33]. The choice of fourth-generation quinolones, the duration of treatment, and the difference in PPI also influenced the pooled eradication rates of therapies containing fourth-generation quinolones.

The subgroup analyses of antibiotic species conducted in this study demonstrated that regimens containing moxifloxacin were superior to those not containing moxifloxacin (84.3% vs 71.9%). This finding might be consistent with a previous systematic

review^[45], but the eradication rate was still less than 85% by ITT analysis. The main reason was that the resistance rate of *H. pylori* to moxifloxacin was higher, even reaching up to 27.0% when analyzed by the E-test^[31]. This phenomenon reminds us that it is best to conduct a susceptibility test to choose antibiotics reasonably.

We also conducted subgroup analysis by region. The eradication rate of fourth-generation quinolone treatments in Europe was much higher than that in Asia (89.1% vs 76.7%). This difference may be due to the low utilization rate of antibiotics in Europe^[8]. In Asia, the abuse of antibiotics is very common, which leads to a high drug resistance rate of *H. pylori*. According to a multiregion prospective 7-year study by Liu *et al.*^[46], the prevalence of *H. pylori* after moxifloxacin treatment was 17.2%. Resistance to moxifloxacin was reported to be similar to that of levofloxacin, ranging from 14.9% to 20.0% in Turkey^[29]. The increasing antibiotic resistance rate makes the eradication of *H. pylori* more difficult.

The rate of incidence of adverse events in the control groups was higher than that in the experimental groups. The pooled OR (1.874) indicated that the use of fourth-generation quinolones in the treatment of *H. pylori* infection can reduce the incidence of adverse

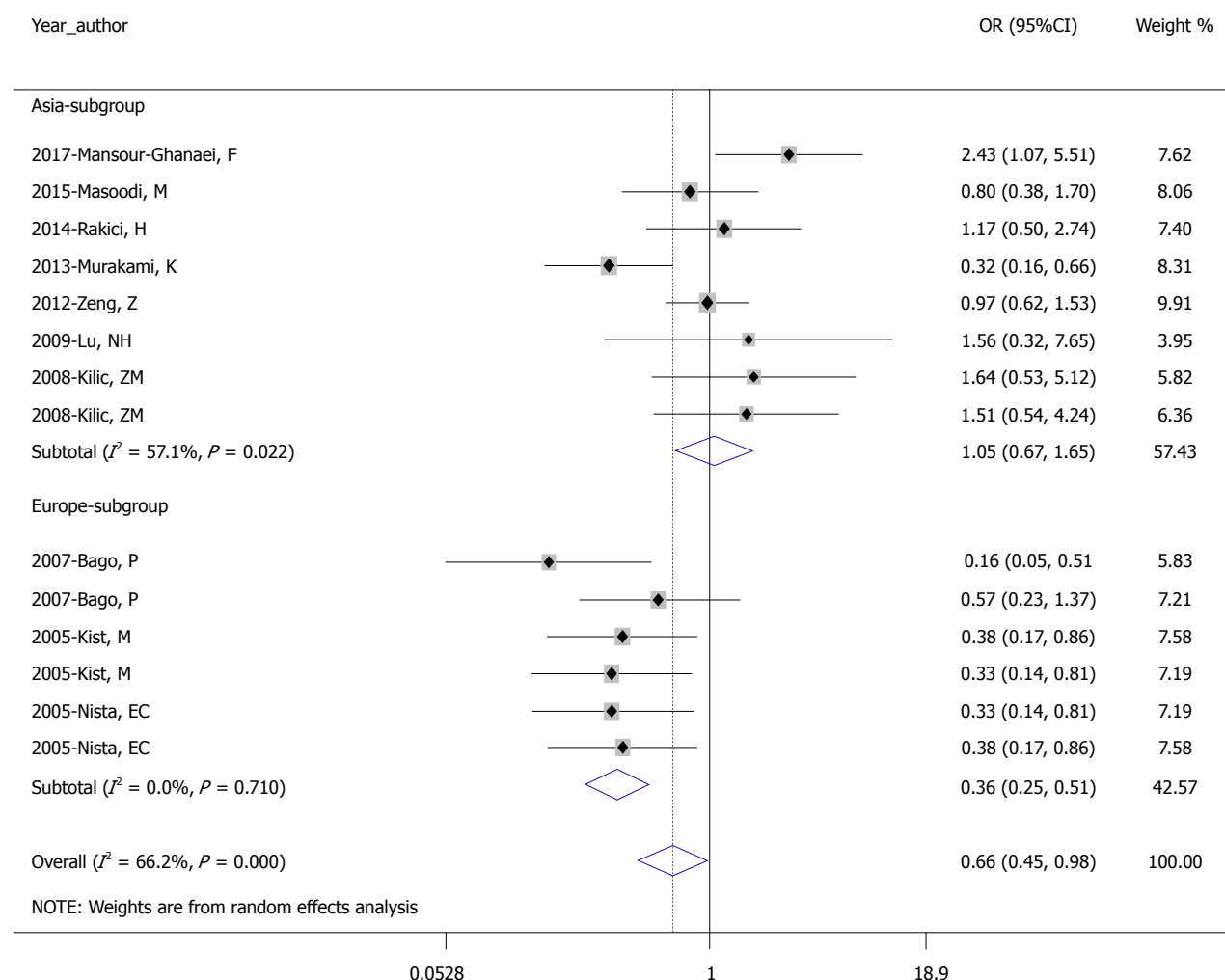


Figure 4 Forest plot of the sub-analysis according to region (intention-to-treat analysis).

reactions. This result indicates that therapies containing fourth-generation quinolones are safer.

The main limitation of this meta-analysis is potential biases. On the one hand, the largest number of studies was conducted using moxifloxacin; only one study used sitafloxacin, and two used gemifloxacin. This selection had a certain effect on the pooled eradication rate and may also be a particularly important issue in the use of a single antibiotic to eradicate *H. pylori* for clinical treatment. On the other hand, all included studies were performed in Europe and Asia, with no studies conducted in Africa or America. Because *H. pylori* infection occurs worldwide, our results may not be appropriate for global generalization. These two factors lead to the bias of conclusion. In addition, most of the studies in our meta-analysis had problems with concealment of allocation and blinding, which caused the selection bias. The restrictions on the language of publication also imply other bias, and thus our meta-analysis may not reflect all the outcomes.

Our analysis also implied other limitations. Most articles reporting a control arm were conducted using clarithromycin; our analysis is therefore especially

lacking detailed data on levofloxacin. Two of the 10 included studies were abstracts, generating concerns regarding the data extraction and quality assessment of these studies and affecting the reliability of our results.

In conclusion, this meta-analysis indicates that therapies containing fourth-generation quinolones can achieve a higher eradication rate of *H. pylori* infection, but the eradication rate remains poor. In the absence of other drug options or in cases of patient allergy to penicillin, such regimens might be considered as a rescue treatment based on antimicrobial susceptibility testing. Further investigation is necessary to draw more solid conclusions about the use of fourth-generation quinolones in the treatment of *H. pylori* infection. In addition, we will study more effective therapies for *H. pylori* infection if necessary.

ARTICLE HIGHLIGHTS

Research background

The resistance of *Helicobacter pylori* (*H. pylori*) to antibiotics is increasing and often leads to the failure of eradication treatment. Recent studies have reported that therapies containing fourth-generation quinolones remain effective against antibiotic-resistant *H. pylori*. However, the efficacy and safety of these therapies require further study. This is the first meta-analysis comparing the curative

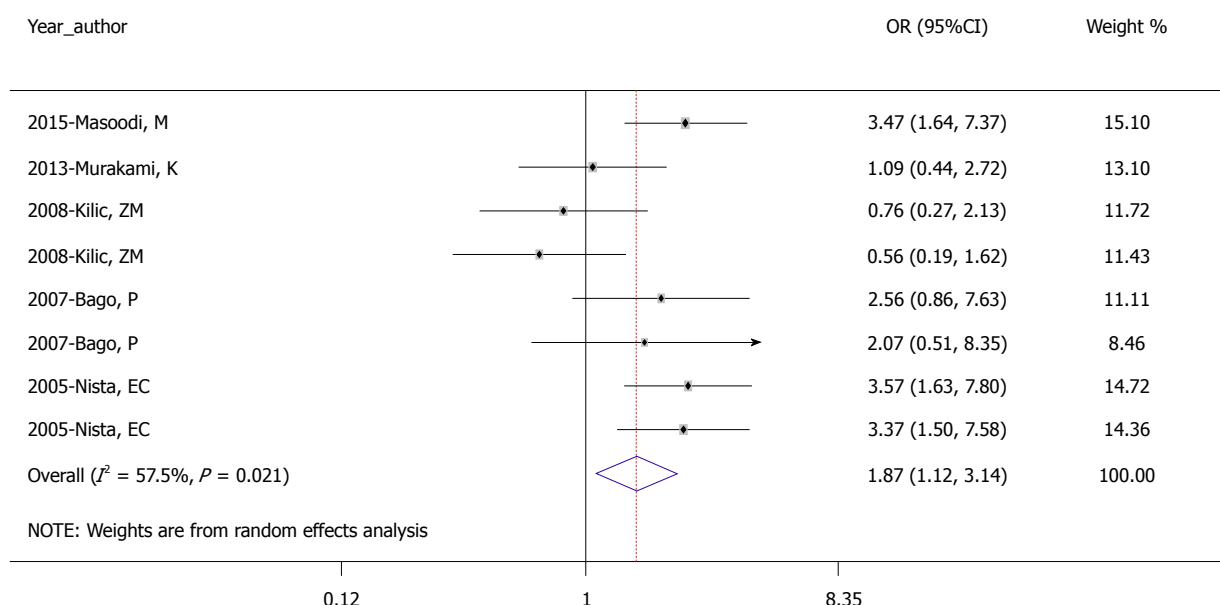


Figure 5 Forest plot of the side effects of the therapies containing fourth-generation quinolones vs the therapies containing non-fourth-generation quinolones.

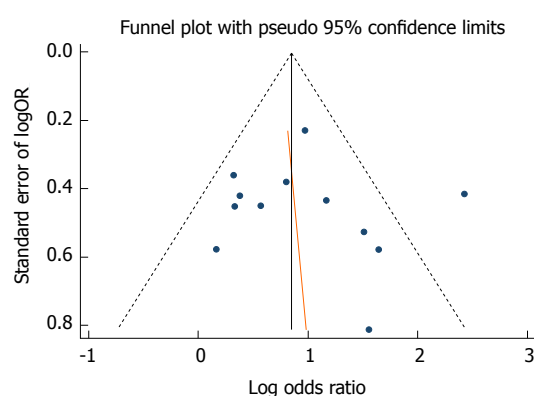


Figure 6 Funnel plot of the therapies containing non-fourth-generation quinolones vs the therapies containing fourth-generation quinolones (intention-to-treat analysis).

effect of fourth-generation quinolones with that of other therapies in regard to eradicating *H. pylori*.

Research motivation

In the Maastricht IV and Maastricht V Consensus Reports, levofloxacin-based therapy is recommended when the first treatment fails. Therapies containing fourth-generation quinolones are not mentioned. Our meta-analysis focused on eradication rates, side effects and compliance of therapies containing fourth-generation quinolones when compared with therapies using non-fourth-generation quinolones.

Research objectives

This meta-analysis aimed to clarify the effect of fourth-generation quinolones on the eradication of *H. pylori* infection and provide some evidence for clinical practice.

Research methods

The meta-analysis was conducted according to the PRISMA criteria. We searched the PubMed, EMBASE, and Cochrane Library databases. The outcome was to calculate the pooled eradication rate and therapy-related side effects among the

trials, comparing the control and experimental groups. We calculated the odds ratio of each trial for the primary measure. The odds ratios were presented with 95% confidence intervals, and a P -value < 0.05 was considered significant. This methodology was also performed for subgroup analysis.

Research results

Available data from 10 studies showed that treatment with a fourth-generation quinolone could achieve a higher *H. pylori* eradication rate and decrease the side effects, but the eradication rate is less than acceptable. Fourth-generation quinolones can significantly improve the eradication rate in Europe but not in Asia.

Research conclusions

Quinolone resistance increases with age and duration of use. It is essential for practitioners to use quinolone antibiotics in the clinic reasonably. This study comprehensively analyzed the role of fourth-generation quinolone in the treatment of *H. pylori* infection. Our results suggested that fourth-generation quinolones are not ideal for eradication of *H. pylori*. Treatment based on antibiotic susceptibility testing might be more valid and obtain a higher rate of eradication of *H. pylori* infection, particularly in areas where resistance to antibiotics develops rapidly.

Research perspectives

According to reports that mutations at positions 87 and 91 of *gyrA* are the main cause of *H. pylori* resistance to fourth-generation quinolones, we will continue to pay attention to the resistance rate to fourth-generation quinolones globally. We will also focus on rapid genotyping methods, such as detecting *gyrA* mutations in *H. pylori*. Further studies of sitafloxacin, gemifloxacin, and gatifloxacin are imperative to draw more solid conclusions about the use of fourth-generation quinolones for the eradication of *H. pylori* infection.

REFERENCES

- 1 Liou JM, Lin JT, Chang CY, Chen MJ, Cheng TY, Lee YC, Chen CC, Sheng WH, Wang HP, Wu MS. Levofloxacin-based and clarithromycin-based triple therapies as first-line and second-line treatments for *Helicobacter pylori* infection: a randomised comparative trial with crossover design. *Gut* 2010; **59**: 572-578 [PMID: 20427390 DOI: 10.1136/gut.2009.198309]
- 2 Molina-Infante J, Romano M, Fernandez-Bermejo M, Federico A, Gravina AG, Pozzati L, Garcia-Abadia E, Vinagre-Rodriguez G, Martinez-Alcala C, Hernandez-Alonso M, Miranda A,

- Iovene MR, Pazos-Pacheco C, Gisbert JP. Optimized nonbismuth quadruple therapies cure most patients with *Helicobacter pylori* infection in populations with high rates of antibiotic resistance. *Gastroenterology* 2013; **145**: 121-128.e1 [PMID: 23562754 DOI: 10.1053/j.gastro.2013.03.050]
- 3 **Song M**, Ang TL. Second and third line treatment options for *Helicobacter pylori* eradication. *World J Gastroenterol* 2014; **20**: 1517-1528 [PMID: 24587627 DOI: 10.3748/wjg.v20.i6.1517]
 - 4 **Wang B**, Lv ZF, Wang YH, Wang H, Liu XQ, Xie Y, Zhou XJ. Standard triple therapy for *Helicobacter pylori* infection in China: a meta-analysis. *World J Gastroenterol* 2014; **20**: 14973-14985 [PMID: 25356059 DOI: 10.3748/wjg.v20.i40.14973]
 - 5 **Malfertheiner P**, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ. Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. *Gut* 2007; **56**: 772-781 [PMID: 17170018 DOI: 10.1056/NEJMcpl001110]
 - 6 **Molina-Infante J**, Pazos-Pacheco C, Vinagre-Rodriguez G, Perez-Gallardo B, Dueñas-Sadornil C, Hernandez-Alonso M, Gonzalez-Garcia G, Mateos-Rodriguez JM, Fernandez-Bermejo M, Gisbert JP. Nonbismuth quadruple (concomitant) therapy: empirical and tailored efficacy versus standard triple therapy for clarithromycin-susceptible *Helicobacter pylori* and versus sequential therapy for clarithromycin-resistant strains. *Helicobacter* 2012; **17**: 269-276 [PMID: 22759326 DOI: 10.1111/j.1523-5378.2012.00947.x]
 - 7 **Mitui M**, Patel A, Leos NK, Doern CD, Park JY. Novel *Helicobacter pylori* sequencing test identifies high rate of clarithromycin resistance. *J Pediatr Gastroenterol Nutr* 2014; **59**: 6-9 [PMID: 25222804 DOI: 10.1097/MPG.0000000000000380]
 - 8 **Megraud F**, Coenen S, Versporten A, Kist M, Lopez-Brea M, Hirschl AM, Andersen LP, Goossens H, Glupczynski Y; Study Group participants. *Helicobacter pylori* resistance to antibiotics in Europe and its relationship to antibiotic consumption. *Gut* 2013; **62**: 34-42 [PMID: 22580412 DOI: 10.1136/gutjnl-2012-302254]
 - 9 **Horiki N**, Omata F, Uemura M, Suzuki S, Ishii N, Iizuka Y, Fukuda K, Fujita Y, Katsurahara M, Ito T, Cesar GE, Imoto I, Takei Y. Annual change of primary resistance to clarithromycin among *Helicobacter pylori* isolates from 1996 through 2008 in Japan. *Helicobacter* 2009; **14**: 86-90 [PMID: 19751432 DOI: 10.1111/j.1523-5378.2009.00714.x]
 - 10 **Thung I**, Aramin H, Vavinskaya V, Gupta S, Park JY, Crowe SE, Valasek MA. Review article: the global emergence of *Helicobacter pylori* antibiotic resistance. *Aliment Pharmacol Ther* 2016; **43**: 514-533 [PMID: 26694080 DOI: 10.1111/apt.13497]
 - 11 **Malfertheiner P**, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T, El-Omar EM, Kuipers EJ; European *Helicobacter* Study Group. Management of *Helicobacter pylori* infection--the Maastricht IV/ Florence Consensus Report. *Gut* 2012; **61**: 646-664 [PMID: 22491499 DOI: 10.1136/gutjnl-2012-302084]
 - 12 **Malfertheiner P**, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European *Helicobacter* and Microbiota Study Group and Consensus panel. Management of *Helicobacter pylori* infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]
 - 13 **Saracino IM**, Zullo A, Holton J, Castelli V, Fiorini G, Zaccaro C, Ridola L, Ricci C, Gatta L, Vaira D. High prevalence of primary antibiotic resistance in *Helicobacter pylori* isolates in Italy. *J Gastrointest Liver Dis* 2012; **21**: 363-365 [PMID: 23256118]
 - 14 **Gao W**, Cheng H, Hu F, Li J, Wang L, Yang G, Xu L, Zheng X. The evolution of *Helicobacter pylori* antibiotics resistance over 10 years in Beijing, China. *Helicobacter* 2010; **15**: 460-466 [PMID: 21083752 DOI: 10.1111/j.1523-5378.2010.00788.x]
 - 15 **Shiota S**, Reddy R, Alsarraj A, El-Serag HB, Graham DY. Antibiotic Resistance of *Helicobacter pylori* Among Male United States Veterans. *Clin Gastroenterol Hepatol* 2015; **13**: 1616-1624 [PMID: 25681693 DOI: 10.1016/j.cgh.2015.02.005]
 - 16 **Jeong MH**, Chung JW, Lee SJ, Ha M, Jeong SH, Na S, Na BS, Park SK, Kim YJ, Kwon KA, Ko KI, Jo Y, Hahm KB, Jung HY. [Comparison of rifabutin- and levofloxacin-based third-line rescue therapies for *Helicobacter pylori*]. *Korean J Gastroenterol* 2012; **59**: 401-406 [PMID: 22735872 DOI: 10.4166/kjg.2012.59.6.401]
 - 17 **Mah FS**. Fourth-generation fluoroquinolones: new topical agents in the war on ocular bacterial infections. *Curr Opin Ophthalmol* 2004; **15**: 316-320 [PMID: 15232471 DOI: 10.1097/00055735-200408000-00007]
 - 18 **Kłosińska-Szmurło E**, Grudzień M, Betlejewska-Kielak K, Pluciński F, Biernacka J, Mazurek AP. Physicochemical properties of lomefloxacin, levofloxacin, and moxifloxacin relevant to the biopharmaceutics classification system. *Acta Chim Slov* 2014; **61**: 827-834 [PMID: 25551723]
 - 19 **Hirata Y**, Serizawa T, Shichijo S, Suzuki N, Sakitani K, Hayakawa Y, Yamada A, Koike K. Efficacy of triple therapy with esomeprazole, amoxicillin, and sitafloxacin as a third-line *Helicobacter pylori* eradication regimen. *Int J Infect Dis* 2016; **51**: 66-69 [PMID: 27590563 DOI: 10.1016/j.ijid.2016.08.019]
 - 20 **Mahmoudi L**, Farshad S, Seddigh M, Mahmoudi P, Ejtehadi F, Niknam R. High efficacy of gemifloxacin-containing therapy in *Helicobacter Pylori* eradication: A pilot empirical second-line rescue therapy. *Medicine (Baltimore)* 2016; **95**: e4410 [PMID: 27759625 DOI: 10.1097/MD.00000000000004410]
 - 21 **Sugimoto M**, Sahara S, Ichikawa H, Kagami T, Uotani T, Furuta T. High *Helicobacter pylori* cure rate with sitafloxacin-based triple therapy. *Aliment Pharmacol Ther* 2015; **42**: 477-483 [PMID: 26075959 DOI: 10.1111/apt.13280]
 - 22 **Gisbert JP**, Romano M, Molina-Infante J, Lucendo AJ, Medina E, Modolell I, Rodríguez-Tellez M, Gomez B, Barrio J, Perona M, Ortuño J, Ariño I, Domínguez-Muñoz JE, Perez-Aisa Á, Bermejo F, Domínguez JL, Almela P, Gomez-Camarero J, Millastre J, Martín-Noguero E, Gravina AG, Martorano M, Miranda A, Federico A, Fernandez-Bermejo M, Angueira T, Ferrer-Barcelo L, Fernández N, Marin AC, McNicholl AG. Two-week, high-dose proton pump inhibitor, moxifloxacin triple *Helicobacter pylori* therapy after failure of standard triple or non-bismuth quadruple treatments. *Dig Liver Dis* 2015; **47**: 108-113 [PMID: 25454706 DOI: 10.1016/j.dld.2014.10.009]
 - 23 **Chung KH**, Dong HL, Kim N, Shin CM, Jin HH, Sang HL, Lee D, Hong SO, Jin EH. Su1696 Efficacy of Second-Line Treatment for *Helicobacter pylori* Infection: Moxifloxacin-Containing Triple Therapy vs. Bismuth-Containing Quadruple Therapy. *Gastroenterology* 2012; **142**: S-483-S-484 [DOI: 10.1016/S0016-5085(12)61839-3]
 - 24 **Moher D**, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med* 2009; **6**: e1000097 [PMID: 19621072 DOI: 10.1371/journal.pmed.1000097]
 - 25 **Moher D**, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Int J Surg* 2010; **8**: 336-341 [PMID: 20171303 DOI: 10.1016/j.ijsu.2010.02.007]
 - 26 **Higgins JP**, Green S. *Cochrane Handbook for Systematic Reviews of Interventions* Version 5.1.0 [updated March 2011]. The Cochrane Collaboration, 2011
 - 27 **Mansour-Ghanaei F**, Pedarpour Z, Shafaghi A, Joukar F. Clarithromycin versus Gemifloxacin in Quadruple Therapeutic Regimens for *Helicobacter Pylori* Infection Eradication. *Middle East J Dig Dis* 2017; **9**: 100-106 [PMID: 28638586 DOI: 10.15171/mejdd.2017.58]
 - 28 **Masoodi M**, Talebi-Taher M, Tabatabaie K, Khaleghi S, Faghihi AH, Agah S, Asadi R. Clarithromycin vs. Gemifloxacin in Quadruple Therapy Regimens for Empiric Primary Treatment of *Helicobacter pylori* Infection: A Randomized Clinical Trial. *Middle East J Dig Dis* 2015; **7**: 88-93 [PMID: 26106468]
 - 29 **Rakici H**, Ayaz T, Akdogan RA, Bedir R. Comparison of levofloxacin- and moxifloxacin-based triple therapies with standard treatment in eradication of *Helicobacter pylori* as first-line therapy. *Digestion* 2014; **90**: 261-264 [PMID: 25547786 DOI: 10.1159/000369788]

- 30 **Murakami K**, Furuta T, Ando T, Nakajima T, Inui Y, Oshima T, Tomita T, Mabe K, Sasaki M, Suganuma T, Nomura H, Satoh K, Hori S, Inoue S, Tomokane T, Kudo M, Inaba T, Take S, Ohkusa T, Yamamoto S, Mizuno S, Kamoshida T, Amagai K, Iwamoto J, Miwa J, Kodama M, Okimoto T, Kato M, Asaka M; Japan GAST Study Group. Multi-center randomized controlled study to establish the standard third-line regimen for *Helicobacter pylori* eradication in Japan. *J Gastroenterol* 2013; **48**: 1128-1135 [PMID: 23307042 DOI: 10.1007/s00535-012-0731-8]
- 31 **Zeng Z**, Lv N, Hu F, Si J, Wu K, Jiang B, Liu W, Zhang J, Chen M, Hu P. Moxifloxacin-based triple therapy for *Helicobacter pylori* eradication: A multicenter randomized parallel-controlled study. *J Gastroenterol Hepatol* 2012; **27**: 3
- 32 **Lu NH**, Xie Y, Zhu X, Chen YX, Ma JH, He XX. Eradication therapy for *Helicobacter pylori* infection in patients with duodenal ulcers based on moxifloxacin triple therapy: a randomized controlled trial. *J Gastroenterol Hepatol* 2009; **24**: A15-A15
- 33 **Kiliç ZM**, Köksal AS, Cakal B, Nadir I, Ozin YO, Kuran S, Sahin B. Moxifloxacin plus amoxicillin and ranitidine bismuth citrate or esomeprazole triple therapies for *Helicobacter pylori* infection. *Dig Dis Sci* 2008; **53**: 3133-3137 [PMID: 18465244 DOI: 10.1007/s10620-008-0285-z]
- 34 **Bago P**, Vcev A, Tomic M, Rozankovic M, Marusić M, Bago J. High eradication rate of *H. pylori* with moxifloxacin-based treatment: a randomized controlled trial. *Wien Klin Wochenschr* 2007; **119**: 372-378 [PMID: 17634896 DOI: 10.1007/s00508-007-0807-2]
- 35 **Kist M**. How effective is moxifloxacin for the first-line treatment of patients with *Helicobacter pylori* infection? *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 450-451 [PMID: 16224475 DOI: 10.1038/ncpgasthep0288]
- 36 **Nista EC**, Candelli M, Zocco MA, Cazzato IA, Cremonini F, Ojetti V, Santoro M, Finizio R, Pignataro G, Cammarota G, Gasbarrini G, Gasbarrini A. Moxifloxacin-based strategies for first-line treatment of *Helicobacter pylori* infection. *Aliment Pharmacol Ther* 2005; **21**: 1241-1247 [PMID: 15882245 DOI: 10.1111/j.1365-2036.2005.02412.x]
- 37 **Thamphiwatana S**, Gao W, Obonyo M, Zhang L. In vivo treatment of *Helicobacter pylori* infection with liposomal linolenic acid reduces colonization and ameliorates inflammation. *Proc Natl Acad Sci U S A* 2014; **111**: 17600-17605 [PMID: 25422427 DOI: 10.1073/pnas.1418230111]
- 38 **Apostolopoulos P**, Koumoutsos I, Ekmektzoglou K, Dogantzis P, Vlachou E, Kalantzis C, Tsiouris P, Alexandrakakis G. Concomitant versus sequential therapy for the treatment of *Helicobacter pylori* infection: a Greek randomized prospective study. *Scand J Gastroenterol* 2016; **51**: 145-151 [PMID: 26435055 DOI: 10.3109/00365521.2015.1079646]
- 39 **Alhoeei S**, Tirgar Fakheri H, Hosseini V, Maleki I, Taghvaei T, Valizadeh SM, Bari Z. A Comparison between Hybrid and Concomitant Regimens for *Helicobacter Pylori* Eradication: A Randomized Clinical Trial. *Middle East J Dig Dis* 2016; **8**: 219-225 [PMID: 27698972 DOI: 10.1517/mejdd.2016.24]
- 40 **Egan BJ**, Marzio L, O'Connor H, O'Morain C. Treatment of *Helicobacter pylori* infection. *Helicobacter* 2008; **13** Suppl 1: 35-40 [PMID: 18783520 DOI: 10.1111/j.1523-5378.2008.00639.x]
- 41 **Kwon YH**, Kim N, Lee JY, Choi YJ, Yoon K, Nam RH, Suh JH, Lee JW, Lee DH. Comparison of the efficacy of culture-based tailored therapy for *Helicobacter pylori* eradication with that of the traditional second-line rescue therapy in Korean patients: a prospective single tertiary center study. *Scand J Gastroenterol* 2016; **51**: 270-276 [PMID: 26452405 DOI: 10.3109/00365521.2015.1095352]
- 42 **Moore RA**, Beckthold B, Wong S, Kureishi A, Bryan LE. Nucleotide sequence of the *gyrA* gene and characterization of ciprofloxacin-resistant mutants of *Helicobacter pylori*. *Antimicrob Agents Chemother* 1995; **39**: 107-111 [PMID: 7695290 DOI: 10.1128/AAC.39.1.107]
- 43 **Lee JW**, Kim N, Nam RH, Park JH, Kim JM, Jung HC, Song IS. Mutations of *Helicobacter pylori* associated with fluoroquinolone resistance in Korea. *Helicobacter* 2011; **16**: 301-310 [PMID: 21762270 DOI: 10.1111/j.1523-5378.2011.00840.x]
- 44 **Graham DY**, Lee YC, Wu MS. Rational *Helicobacter pylori* therapy: evidence-based medicine rather than medicine-based evidence. *Clin Gastroenterol Hepatol* 2014; **12**: 177-186.e3; Discussion e12-e13 [PMID: 23751282 DOI: 10.1016/j.cgh.2013.05.028]
- 45 **Zhang G**, Zou J, Liu F, Bao Z, Dong F, Huang Y, Yin S. The efficacy of moxifloxacin-based triple therapy in treatment of *Helicobacter pylori* infection: a systematic review and meta-analysis of randomized clinical trials. *Braz J Med Biol Res* 2013; **46**: 607-613 [PMID: 23903685 DOI: 10.1590/1414-431X20132817]
- 46 **Liu DS**, Wang YH, Zeng ZR, Zhang ZY, Lu H, Xu JM, Du YQ, Li Y, Wang JB, Xu SP, Chen Y, Lan CH, Cheng H, Jiang MD, Zhang LX, Huo LJ, Chen SY, Zhang GX, Wu KC, Zhu X, Chen YX, Zhu Y, Shu X, Xie Y, Lu NH. Primary antibiotic resistance of *Helicobacter pylori* in Chinese patients: a multiregion prospective 7-year study. *Clin Microbiol Infect* 2018; **24**: 780.e5-780.e8 [PMID: 29138101 DOI: 10.1016/j.cmi.2017.11.010]

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