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Ki-67 and Bax expression in esophageal mucosa might have implications in ablative therapies for Barrett's esophagus, dysplasia, and adenocarcinoma

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Dear Editor,

We read with considerable interest the paper by Lewis et al. [1], who concluded that the buried glandular tissue of postablation patients with Barrett's esophagus (BE), and mainly with esophageal high-grade dysplasia (HGD) and esophageal adenocarcinoma (EA), still exhibits a higher superficial or deep expression of the oncogene Ki-67 than normal esophageal epithelium, thereby having potential implications for the follow-up management of these patients, because it is unclear whether the true risk of neoplastic progression is adequately reduced. Although the authors measured pre- and postablation levels of cyclo-oxygenase (COX)-2 (indicating increased neoplastic potential in BE), Ki-67 (indicating cellular proliferation), and Bcl-2 protein (indicating resistance to apoptosis), they did not measure a simultaneous prEApoptotic oncoprotein, such as Bax, which opposes the effects of Bcl-2 and Ki-67 oncogenes, possibly associated more robustly with BE–HGD–EA sequence, and thus having a greater impact on the follow-up management of these patients.

In this regard, BE-associated EA is now the most common esophageal malignancy in Western countries; in the United States and England, the incidence is increasing faster than any other cancer [2]. EA demonstrates extremely rapid progression [3], and the mortality rates from esophageal cancer per 100,000 person-years are approximately 8 in the United States, 13 in Japan, 17 in France, and 36 in rural China. On the other hand, mortality from esophageal cancer in Greece is among the lowest in the world (3.5 per 100,000 person-years; WHO 1995). It has been argued that populations with unusually low disease rates can provide valuable insights into the etiologic processes [4].

Previous studies have attempted to identify risk factors in dietary, lifestyle, and medical conditions of the Greek population. Although some inverse association was found between EA and vegetables and fruits consumed in large quantities, no clear-cut answer has emerged as to why the incidence of EA is so low in Greece [5]. Therefore, we tried to provide an explanation for the low incidence of EA in Greece through molecular-based ethnic differentiations. The role of oncogene Ki-67, promoting cell proliferation and oncogenesis, and the Bcl-2 and Bax proteins, important regulators of programmed cell death (apoptosis), was investigated in a Greek cohort of BE patients and gastroesophageal reflux disease (GERD) controls [6].

GERD is well-recognized, premalignant condition that has a pivotal role in BE–HGD–EA sequence [7, 8]. Specifically, of 270 patients who underwent upper gastrointestinal endoscopic and histologic evaluation due to GERD symptoms, 31 patients with BE (typical salmon-pink lesions) and 21 GERD controls were ultimately included in the study. Esophageal mucosa biopsy specimens also were evaluated immunohistochemically for Ki-67, Bax, and Bcl-2 expressions. Increased expression of Ki-67 was observed in BE patients compared with GERD controls ($P = 0.01$). Increased expression of Bax was noticed in the total BE patients compared with GERD controls ($P < 0.001$). Bcl-2 expression did not differ between the two groups.

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Our findings indicate a possible existence of a balance between cell proliferation (indicated by Ki-67 increased expression) and apoptosis (indicated by Bax protein overexpression), thereby providing an equilibrium between cell apoptosis and cell proliferation. This may partly explain the low EA incidence in Greece.

In view of the aforementioned data, it would be interesting to know whether the authors have considered the possible implication of the balance or imbalance between Ki-67 and Bax expression as a more representative index for the follow-up management of patients associated with BE–HGD–EA sequence.

Another, still controversial, issue, *Helicobacter pylori* infection may be involved in the GERD–BE–EA sequence and its eradication might inhibit the progress of this sequence [8–14]. When *H. pylori* colonizes the esophagus, the severity of esophageal inflammation and the incidence of BE and EA are increased [13]. In this respect, our own and other relative data show that *H. pylori* is frequent in GERD, even in nonendoscopic reflux disease and BE [9–11], and *H. pylori* eradication leads to a better control of GERD symptoms and improves esophagitis [12]. In particular, *H. pylori* infection induced nuclear factor κB and COX-2 expression in esophageal epithelial cells, playing a role in inflammation and tumorigenesis in the esophagus [4]; higher levels of COX-2 expression and prostaglandin E_2 were detected in rats with esophageal *H. pylori* colonization [13]. Therefore, it would be interesting to know whether the authors have considered any relationship between *H. pylori* infection and pre- and postablation levels of COX-2, Ki-67, and/or Bcl-2.

Conflict of interests None declared.

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Letter to the Editor

Helicobacter pylori's potential association with epilepsy

Dear Editor,

We have read with interest the study by Asadi-Pooya et al. who concluded that the rate of *Helicobacter pylori* infection (*Hp*-I) was not higher in patients with epilepsy compared to healthy individuals.[1] However, due to problems mainly encountered during recruitment of the patients and controls of this study, the conclusion of this trial might be not reasonable.

It has been reported that the prevalence of *Hp*-I in the city of Shiraz, where the study by Asadi-Pooya et al. was conducted, is very high,[2,3] regardless of the socioeconomic status, an already established significant factor affecting *Hp* prevalence in the European countries. This means that to prove a difference in *Hp* prevalence between any two groups in Shiraz would require several hundreds or even a few thousands of participants. Instead, Asadi-Pooya et al. presented their results based on a small number of patients and controls (34 and 33, respectively) and therefore the power of their study was too low. Specifically, the study groups were not age-matched. It seems that the control group was about 10 years older than the idiopathic generalized epilepsy (IGE) group and 2–3 years older than the temporal lobe epilepsy (TLE) group. *Hp* prevalence is higher in older individuals, and this explains why *Hp*-I was detected in 72.7% in the control group compared with 61.8% in the IGE and 50% of the TLE group. These differences did not reach a statistical significance due to the very small number of patients recruited, but it is almost certain that the *p* value equal to 0.068 reported would have been significant if the authors had recruited just a few more patients. In that case the control healthy group would have a higher *Hp* prevalence than the epileptic patients, leading to the paradox conclusion that *Hp* might protect against epilepsy.

On the other hand, recent data suggest a probable association between *Hp*-I and epilepsy, especially with poor prognosis.[4] In this respect, we speculate that *Hp*-I, by inducing proinflammatory cytokine production and blood–brain barrier (BBB) disruption,[5] also mentioned by the aurhors,[1] may lead to neuroinflammation and neuronal damage in epilepsy thereby triggering seizures' induction and epilepsy progression.[5]

Conflict of interest

None.

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LETTER TO THE EDITORS

A rebuttal to the potential anti-tumour benefit of *Helicobacter pylori*-induced neutrophil-activating protein

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Dear Editors,

In their paper, Codolo et al. [1] concluded that *Helicobacter pylori* (*HP*) neutrophil-activating protein (*HP*-NAP), by activating a cytotoxic T helper (Th) 1 response, inhibits the growth of bladder cancer in mice, findings strongly indicating that *HP*-NAP might become a novel therapeutic 'bullet' for the cure of bladder tumours. However, their remarks raise some concerns.

Although *HP*-NAP plays a major role in generating and maintaining the *HP*-associated gastric inflammatory response and, moreover, *HP*-NAP is a promising vaccine candidate against *HP* infection (*HP*-I), concerns regarding its potential drawbacks, particularly neurogenic ones, should be considered before, as the authors considered [1], in vivo studies to assess the *HP*-NAP anti-tumour efficacy in humans are conducted. For instance, possible cross-mimicry between *HP*-NAP and/or bacterial aquaporin (AQP) and neural tissues may be associated with the anti-AQP-4 antibody-related neural damage in multiple sclerosis (MS)/neuromyelitis optica (NMO) patients [2, 3]. In this respect, using histology, recognised as the practical gold standard for the diagnosis of current *HP*-I, we showed a strong association between *HP*-I and MS in a Greek cohort [4]. Current *HP*-I induces irregular humoral and cellular immune responses that, owing to the sharing of homologous epitopes (i.e. molecular mimicry), cross-react with components of nerves, thereby constantly triggering and possibly perpetuating neural tissue damage observed in neurodegenerative diseases [5, 6]. In this regard, *HP* virulence factors promote the release of various chemoattractants/inflammatory mediators including interleukin (IL)-8, IL-12 or interferon (IFN)-γ, mentioned by the authors, and *HP*-NAP, which, as a virulence factor, recruits leucocytes from the vascular lumen, activates neutrophils, monocytes and mast cells [2, 5, 7, 8]. Specifically, a series of factors have been implicated in inducing blood–brain barrier (BBB)/blood nervous barrier (BNB) disruption, including the aforementioned inflammatory mediators (e.g. cytokines and chemokines induced by *HP*-I) and oxidative stress [9]. They increase vascular permeability and BBB/BNB disruption [tumour necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), vascular permeability factor], induce transmigration of leucocytes into the nerve, activation and proliferation of macrophages (IFN-γ) and T cells (IL-1, IL-2) and exert direct myelinotoxic activities (TNF-α and TNF-β). In this respect, Schwann cell induces reactivation of CD4+ T cells, which, by producing TNF-α and IFN-γ, could exacerbate BNB disruption thus playing a role in neuropathies. Intraneural activated T cells cause focal BNB disruption, and, moreover, increased circulating TNF-α might play a role in the BBB/BNB disruption and the pathogenesis of demyelination; *HP* through the release of TNF-α, acting at distance, is involved in BBB/BNB disruption through a mechanism involving matrix metalloproteinase upregulation [9, 10]. *HP*-NAP induces a strong Th1 response, mentioned by the authors [1], a predominant *HP*-specific Th1 response is characterised by a high TNF-α, IFN-γ, IL-2 and IL-12 production leading to apoptotic damage and the Th1 type cytokine TNF-α leads to TNF-α-related apoptotic neuronal cell death in neurodegenerative diseases including, for instance, Alzheimer's disease [11].

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In addition, apart from activated mast cells, VEGF, IL-8, chymase or tryptase (a serine endoprotease released by mast cells) and mast cell growth factor linked to *HP*-I and *HP*-NAP, mast cells themselves can be stimulated by corticotropin-releasing hormone, secreted under stress, to release mediators including histamine, IL-8, tryptase and VEGF, which disrupt the BBB/BNB [9, 10]. In turn, BBB/BNB breakdown could promote entry of immune cell (autoreactive effector CD4+ and CD8+ T cells) infiltrations, activated monocytes infected with *HP,* due to defective autophagy resulting in *HP*-I replication in autophagic vesicles and/or *HP* circulating antibodies into the peripheral and central neurons resulting in the development of neuronal pathologies [9]. Mast cells are located in close proximity to neurons in the peripheral and central nervous systems, signifying their role in normal and aberrant neurodegenerative conditions [9]. Mast cell degranulation is able to secrete potent mediators which could orchestrate neuroinflammation and affect the BBB/BNB integrity, thereby playing a role in neuropathies [9]; BBB disruption permits access of AQP4-specific antibodies and T lymphocytes to the central nervous system, playing a major role in MS/NMO pathogenesis [3]. Therefore, *HP*-NAP and *HP*-I itself, by inducing several mediators, may influence the pathophysiology of neurodegenerative diseases, thus raising possible concerns regarding the *HP*-NAP use as a candidate anti-tumour agent. Nevertheless, relative studies are needed to clarify the aforementioned concerns.

HP is classified as class I carcinogen, and *HP*-NAP may play a role in gastrointestinal oncogenesis; *HP*-NAP antibodies are significantly higher in gastric cancer patients indicating a possible pathogenetic role of *HP*-NAP in gastric carcinogenesis [12]. Specifically, *HP*-NAP may contribute to inflammatory response or carcinogenesis by activating neutrophils, which induce the production of reactive oxygen species (ROS) [12]; ROS can cause a variety of DNA lesions, and they can produce mutations in bacterial and mammalian cells and also induce malignant transformation in cultured fibroblast cell lines [7]. Since *HP*-NAP is a potent stimulant for the production of ROS, mediating damage to DNA and enhancing cell turnover, it appears to be a risk factor for *HP*-associated gastric cancer [12]. Apart from neutrophils and monocytes, *HP*-NAP also activates mast cells [2, 13], and mast cells can be stimulated by corticotropin-releasing hormone, secreted under stress, to release the mentioned mediators which disrupt the BBB permitting metastases of lung and mammary adenocarcinomas [14]. In this regard, *HP*-I appears to be a common denominator of lung, mammary and gastrointestinal tract cancers contributing to the mentioned stress–mast cell–BBB disruption–brain metastases sequence of these malignancies [8].

In view of the aforementioned data, further relative investigation might be important to assess the potential concerns of *HP*-NAP before using it as an innovative therapeutic regimen for the cure of bladder cancer in humans.

Conflict of interest The authors declare that they have no conflict of interest.

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High prevalence of gout with sleep apnea

Introduction

Gout is defined as a disorder in which monosodium urate monohydrate (MSU) crystals are deposited in soft tissues, joints, bone, or renal collecting systems, often aggregated into tophi [1]. When these crystals are deposited in synovium they initiate the immune response which causes the severe joint pain and inflammation of a gout flare. Sometimes they are encased in externally visible or palpable subcutaneous tophi, or they cause urolithiasis. In these situations, the gout is termed symptomatic.

But the formation of tophi is known to occur in tissue that is not externally visible or palpable. In some cases, their presence has been found to interfere with the proper function of organs, such as the heart [2] and the spine [3].

Symptomatic gout has been reported to occur in 2% of the sleep apnea (SA) population [4]. The hypothesis of this correspondence is that gout occurs in a much larger percentage of the sleep apnea population, but in most cases it is asymptomatic gout, in which the tophi are not visible externally or palpable, and there is no gouty joint pain or sign of urilithiasis.

Physiology underlying the hypothesis

Hypoxemia from SA causes gout by several compounding processes. The hypoxemia has been shown [5] to cause a catabolic response in the cells in which adenosine triphosphate begins to disintegrate, which initiates a chain of chemical transitions that culminates irreversibly in the generation of excess uric acid fed into the blood. The hypoxemia also causes hypercapnia and acidosis, which reduces the concentration of uric acid that the blood can hold in solution. Over the long term, the chronic intermittent hypoxemia accelerates reduction of the kidney glomerular filtration rate [6], so that extraction of uric acid from the blood is slowed.

Thus the hypoxemia of SA creates the conditions for a perfect storm in which the MSU crystals precipitate – greater influx of uric acid into the blood, slower efflux from the blood, and reduced storage capacity in the blood. When the serum concentration reaches the tipping point from the abruptly increased level of serum uric acid, MSU precipitates.

This physiology applies to all the SA population, not just the small percentage who suffer from symptomatic gout. This realization forms the basis for the hypothesis of this correspondence that almost all individuals with SA have gout.

Testing the hypothesis

Testing of this hypothesis can be performed on individuals with SA by ultrasonic, magnetic resonance, conventional radiographic, or computed tomography imaging in the area of joints of the extremities, which is where most symptomatic gout flares develop. These imaging techniques have been used [7] to display tophi clustered near joints where symptomatic gout flares have occurred. These techniques may be used effectively with SA patients to gather empirical data for this hypothesis. If the imaging data verify this hypothesis, then imaging of tophi may be useful in the future as a tool to aid in the diagnosis of SA.

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Hpn protein as a mediator between *Helicobacter pylori* infection and Alzheimer's disease in sub-populations worldwide

Dear Editor,

The hypothesis by Ge and Sun [1] proposes that *Helicobacter pylori* (*Hp*)n, a histidine-rich protein abundant in this bacterium that forms amyloid-like oligomers, may be the mediator between *Hp*-infection (*Hp*-I) and Alzheimer's disease (AD), whose close association has been recently discovered by several independent groups from Greece, Italy, Canada and Japan [1].

Specifically, our relative Greek data indicate that *Hp*-I, documented by histology, is more frequent in AD and mild cognitive impairment patients than in controls; *Hp* eradication may positively influence AD manifestations at 2- and 5-year clinical endpoints; *Hp*-specific IgG antibody levels are significantly increased in the cerebrospinal fluid of AD patients and might reflect the AD severity, thereby supporting a role for this common infection in

the pathobiology of AD [2]. Other investigators from Italy and France also reported an association between *Hp*-I and AD [2]. In contrast, Japanese authors reported a lack of association between *Hp*-I and AD in their Japanese cohort [3]. However, both age and sex, found to be associated with AD, were not matched in the two study groups, and thus comparisons between them cannot be expected to establish any firm conclusions. Moreover, the very high *Hp*-I prevalence in the general Japanese population around 70 years old, reported by the authors, and, deductively, in the control group of the study, renders the study underpowered; this study can neither confirm the lack of association between the *Hp*-I and AD in Japanese, nor is it comparable with the European studies indicating such an association [3]. Moreover, because there is as yet no relative data from Canada, the close association, mentioned by the authors, appears to apply in some sub-populations worldwide.

Finally, *Hp*-I, by releasing inflammatory mediators (e.g., cytokines and chemokines) and oxidative stress, leads to blood-brain barrier disruption, which, in turn, apart from the mentioned *Hp*n-related transporters, might promote free entry of *Hp*n proteins into the brain resulting in the development of AD pathologies by inducing amyloid-β-peptide (Aβ) fibril formation or aggregating synergistically with Aβ.

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The addictive hypothesis of suicidal behavior

Major repeaters (individuals with $\geqslant 5$ lifetime suicide attempts) represent between 15% and 20% of suicide attempters [1]. Quite surprisingly, no previous study has considered that major repeaters might be "addicted" to suicidal behavior (SB). But, how can be explained that some individuals attempt suicide more than 80 times [1] without using an addictive paradigm?

Traditionally, addictions were ascribed to substance use [2]. In 1990, Goodman [2], operationalized the concept of behavioral addiction. This supposed a Copernican change because the conceptualization of addictions expanded to embrace behaviors such as internet use, sun-tunning, or even love [3–5].

Behavioral and substance addictions share many characteristics [6]. As with substance dependence, the addiction to SB requires the confluence of a vulnerable individual with a "rewarding object". Indeed, major repeaters carry a heavy mental burden [1]. SB repetition is associated with sociopathy, alcoholism [1], affect dysregulation, poor impulse control, and self-mutilative behavior [7]. On the other hand, SB raises social support [8], produces relief of negative emotions [7], and reduces mental pain [9]. Thus, the release of endogenous opioids, a common substrate in drug addiction, may underlie the addiction to SB [10].

In conclusion, repetitive SB may be addictive. Further research is warranted to confirm this hypothesis.

Disclosure

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Histological Presence of *Helicobacter pylori* Bacteria in the Trabeculum and Iris of Patients with Primary Open-Angle Glaucoma

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Key Words
Helicobacter pylori · Primary open-angle glaucoma · Cresyl violet · Trabeculum

Abstract
Purpose: To investigate the role of *Helicobacter pylori* in primary open-angle glaucoma (POAG) pathophysiology by detecting its presence in eye biopsies of POAG patients during trabeculectomy. ***Patients and Methods:*** Fifty-one consecutive patients who underwent trabeculectomy for POAG not responsive to antiglaucoma therapy, and 35 consecutive anemic controls were examined for *H. pylori* presence mainly by gastric mucosa histology. In POAG patients, eye biopsies were also obtained and stained for *H. pylori* presence in situ. ***Results:*** Forty-three of 51 (84.3%) POAG patients and 17 of 35 (48.6%) controls were tested *H. pylori* positive (p = 0.0004). In 5 *H. pylori*-positive POAG patients, *H. pylori* bacteria were identified in the trabeculum and iris specimens. ***Conclusion:*** For the first time, *H. pylori* bacteria have been detected histologically in eye biopsies of POAG patients.

Introduction

Glaucoma, which is a chronic neurodegeneration of the optic nerve, is currently recognized as an optic neuropathy. After cataract it is the second most frequent cause of world blindness and therefore is of major public health importance [1–3]. A theoretical relationship exists between glaucoma and *Helicobacter pylori* infection: (1) both are diseases of older adults in the developed world [4]; (2) chronic *H. pylori* infection may produce systemic disorders involving vascular tone resulting from the release of vasoactive and proinflammatory substances [4], and (3) *H. pylori* infection is associated with arteriosclerosis-induced increased platelet activation and aggregation [4]. It is thus important to investigate the role of *H. pylori* infection in glaucoma initiation, progression or susceptibility by documenting its qualitative and quantitative presence in this disease.

In 2001, by using histology in gastric biopsy specimens, we documented for the first time a high prevalence of *H. pylori* infection in Greek patients with primary open-angle (POAG) and pseudoexfoliation glaucoma [4]. In 2 subsequent studies, we reported: (1) a beneficial effect of *H. pylori* eradication on glaucoma progression,

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suggesting a possible causal link between the bacterium and glaucoma [5], and (2) increased *H. pylori*-specific IgG antibody concentration in the aqueous humor of POAG patients, the concentration of which correlated with the degree of vertical cupping, possibly indicating the severity of glaucomatous damage [6]. Reports from other ethnic populations have also shown a relationship between glaucoma and *H. pylori* infection [7, 8], although this has not been confirmed by all the relevant studies published so far [9, 10], and thus this association may only apply to a limited subpopulation of glaucoma patients. Similar observations have been made in Korea, China, India, Turkey and Iran [8, 11].

We herein further investigate the additional role of *H. pylori* infection in glaucoma pathophysiology by documenting, for the first time, its presence in incisional biopsy specimens obtained from the trabeculum and iris of patients with POAG during trabeculectomy surgery and stained with cresyl violet.

Patients and Methods

In this new cohort, 51 consecutive patients (33 female, mean age: 71.4 ± 9.2 years, range 50–84 years) underwent trabeculectomy for POAG not responsive to topical antiglaucoma therapy. With the term 'uncontrolled' POAG we define cases where intraocular pressure was 21 mm Hg or more, measured with a calibrated Goldmann applanation tonometer, despite maximum-tolerable glaucoma drug therapy, with evidence of progressing optic neuropathy and visual field defects. The glaucoma patients were included in the study using the following criteria: (1) a history of intraocular pressure of 21 mm Hg or more; (2) typical optic nerve head changes including saucerization, rim thinning or notching in the inferior or superior temporal area of the optic nerve head, or total glaucomatous cupping, and (3) typical visual field loss including a paracentral, arcuate, or Seidel's scotoma or a nasal step. All POAG patients had prior experience with automated perimetry. The visual fields were assessed with the Octopus program G1 (Octopus 500EZ G1, Luterzeag AG, Zurich, Switzerland) by the same perimetrist (G.S.).

We excluded patients with closed-angle glaucoma, secondary glaucoma, previous eye surgery, previous argon laser trabeculoplasty, and patients less than 30 years of age. Patients with myopia or hypermetropia greater than 3 dpt or astigmatism greater than 1 dpt, which could potentially affect our measurements, were also excluded.

Routine complete blood cell count, biochemical tests and electrocardiogram were performed on each patient prior to surgery. Moreover, we established the presence of *H. pylori* through an upper gastrointestinal (GI) endoscopy and histology, or a urea breath test in 8 patients who were deemed not suitable endoscopy candidates or refused to undergo an endoscopy.

Patients were then scheduled for trabeculectomy. Specifically, under a combination of topical and sub-Tenon (Tenon's capsule) anesthesia, an initial pocket was created under the conjunctiva and Tenon's capsule, and the wound bed was treated for several seconds to minutes with mitomycin C-soaked sponges (0.5–0.2 mg/ml) to prevent failure of the filter bleb from scarring by inhibiting fibroblast proliferation. A partial thickness flap with its base at the corneoscleral junction was then made in the sclera after careful cauterization of the flap area. A window opening was created under the flap with a Kelly punch to remove a portion of the sclera, Schlemm's canal and the trabecular meshwork in order to enter the anterior chamber. Iridectomy was also performed to prevent future blockage of the sclerostomy. The scleral flap was then sutured loosely back in place with several sutures. The conjunctiva was closed in a watertight fashion at the end of the procedure.

Tissue samples from the trabeculum, conjunctiva and iris were immediately obtained, were placed in tubes containing 10% formalin and submitted to histologic examination. All specimens were stained with cresyl fast violet stain (for detection of *H. pylori* organisms). The same experienced pathologist (I.V.), who was masked to the other determinants of *H. pylori* status and to the patient groups, assessed all the specimens.

Control subjects consisted of 35 patients without POAG (18 females, mean age of 68.22 ± 8.45 years, range 50–84 years), who underwent upper and lower GI endoscopy for the investigation of mild iron deficiency anemia but in whom endoscopy did not reveal any obvious finding. Exclusion criteria were as described previously [5]. None of the subjects received any treatments such as ferrous sulfate before the diagnosis. *H. pylori* detection methods have also been reported previously [5], except for ^{13}C-urea breath test (Helicobacter INFAI test, Faran Laboratories S.A., Athens, Greece). The ratio of $^{13}CO_2$ to $^{12}CO_2$ in the expired breath was measured by mass spectrometry and expressed as $\delta^{13}CO_2$ (per mil). An automated breath ^{13}C analyzer was used with chromatographic purification and a single inlet isotope ratio mass spectrometer. Excess $\delta^{13}CO_2$ excretion greater than 5 per mil was considered to be positive for *H. pylori* infection [12].

The study was designed according to the principles of the Declaration of Helsinki (1964) and the study protocol was approved by the local ethics committee. All patients gave written informed consent prior to their enrollment in the study.

Statistics

For comparison of the age (years) between POAG patients and control subjects, the independent samples t test was used, whereas for sex the χ^2 test was applied. The latter test was also used to compare the prevalence of *H. pylori* infection between POAG patients and control subjects. Odds ratios and 95% confidence intervals were also calculated between these two groups. Significance was set at $p < 0.05$.

Results

Mean age and sex ratios did not differ between POAG patients and anemic control participants (table 1). Mean duration from establishing glaucoma diagnosis was 7.7 ± 8.6 years, and, despite continuous treatment with topical antiglaucoma therapy, mean intraocular pressure was 24.4 ± 8.9 mm Hg in the right and 23.1 ± 10.2

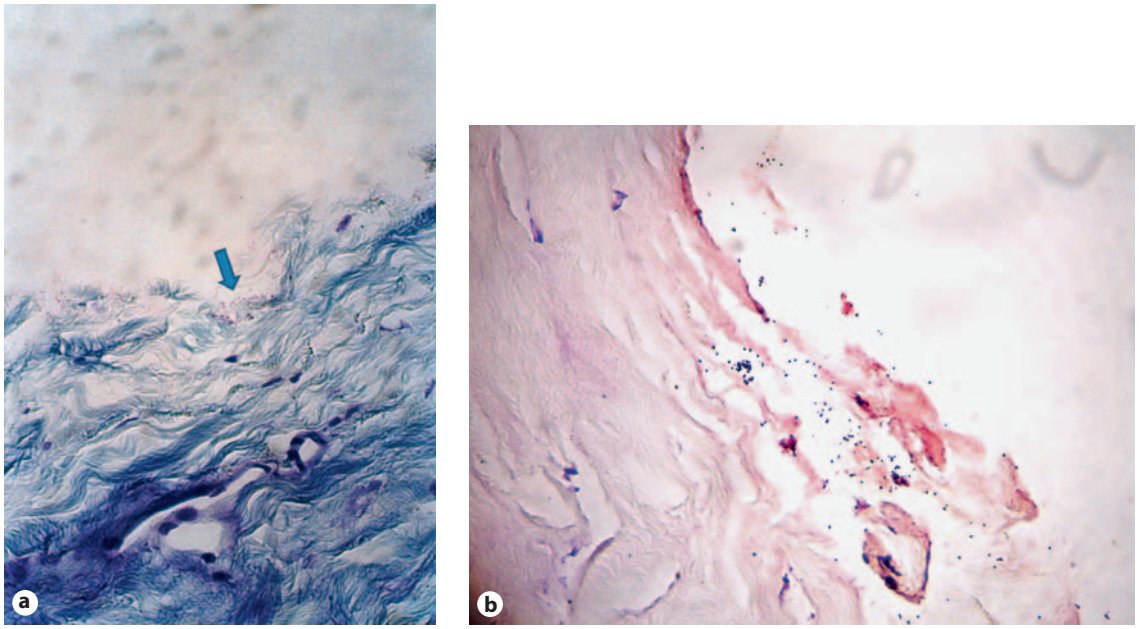

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Fig. 1. a, **b** *H. pylori* bacteria detected histologically on the surface of trabeculum specimens. Cresyl violet stain. ×400.

Table 1. *H. pylori* positivity in POAG patients and anemic controls

Characteristic	POAG patients (n = 51)	Controls (n = 35)	Odds ratio [95% CI]	p value
Age, years				
Mean ± SD	71.4 ± 9.2	68.2 ± 8.5	–	0.06
Range	49–93	50–84		
Sex (m/f)	18/33	18/17	0.5 [0.2–1.3]	0.2
H. pylori presence confirmed histologically or by urea breath test	43/51 (84.3%)	17/35 (48.6%)	5.7 [2.0–16.5]	0.0004
Urea breath test positive for *H. pylori*	4/8 (50%)	–	–	–

– = Not applicable.

mm Hg in the left eye, negatively affecting visual acuity (mean value about 5/10) in both eyes.

Forty-three POAG patients were eligible and they accepted undergoing a selective upper GI endoscopy, of whom 5 had advanced age and serious respiratory comorbidities and were deemed not suitable endoscopy candidates, and the remaining 3 refused to undergo an upper GI endoscopy. All 8 POAG patients in whom biopsies were not obtained underwent a noninvasive urea breath test to detect *H. pylori* infection. All 35 control participants were eligible and also accepted undergoing an upper GI endoscopy.

Forty-three of 51 (84.3%) POAG patients and 17 of 35 (48.6%) anemic control participants were tested positive for *H. pylori* infection by histology or urea breath test (p = 0.0004; table 1). Of the 8 patients for whom a urea breath test was performed, only 4 tested positive for *H. pylori* infection.

In 5 patients, for whom gastric *H. pylori* histology was positive, we managed to identify histologically *H. pylori* bacteria in the trabeculum (fig. 1a, b) and iris specimens (fig. 2) for the first time, using cresyl fast violet stain. In particular, a high density of *H. pylori* microorganisms with their characteristic morphology were identified

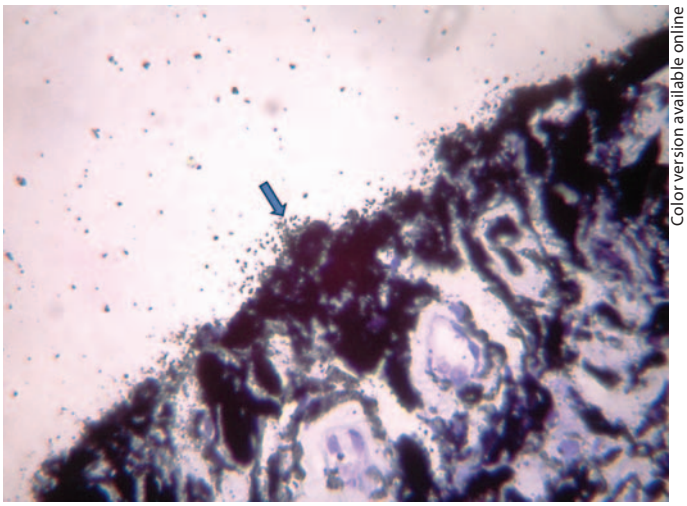

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Fig. 2. *H. pylori* bacteria detected histologically on the surface of iris specimens. Cresyl violet stain. ×400.

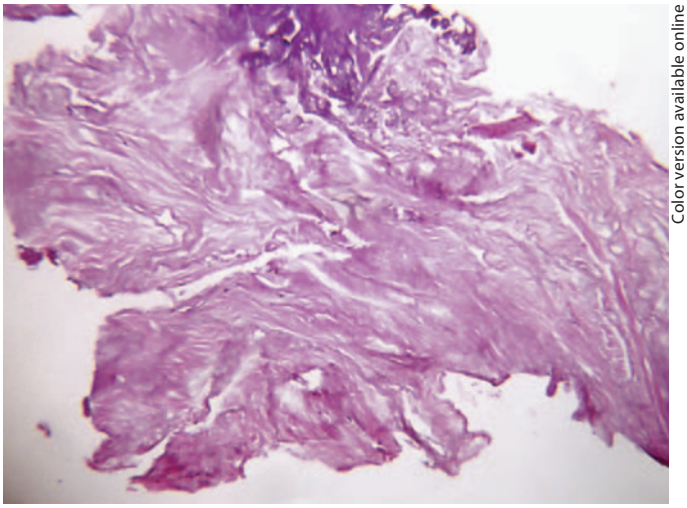

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Fig. 3. Trabeculum specimen without presence of *H. pylori* bacteria. Cresyl violet stain. ×400.

with confidence by cresyl violet staining. These bacteria, having a strong affinity for this stain [13], were present on the surface of the trabeculum and iris areas. There was no evidence of their intratissue colonization. Control slides showing an absence of *H. pylori* bacteria in the trabeculum and iris of POAG patients are shown in figures 3 and 4, respectively, for comparison.

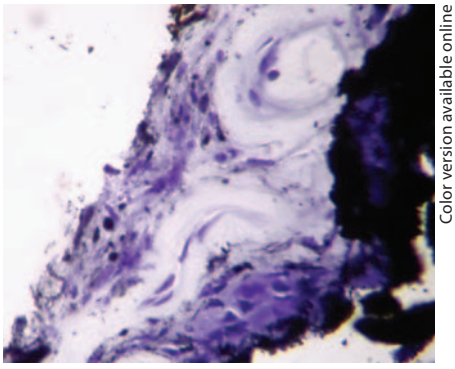

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Fig. 4. Iris specimen without presence of *H. pylori* bacteria. Cresyl violet stain. ×400.

Discussion

Our series have shown, for the first time, the significant finding of the existence of *H. pylori* bacteria in trabeculum and iris specimens of glaucoma patients, thereby further supporting the role for *H. pylori* infection in the pathophysiology of POAG. The reason why *H. pylori* was not found in the trabeculum and iris of all POAG patients who were tested positive for *H. pylori* status could be explained, apart from *H. pylori* absence in the eye, by the very small size of the sample of tissue obtained and submitted for histopathology during the trabeculectomy, or possibly by the standard local antisepsis prior to surgery. Interestingly, it has been shown before that *H. pylori recN* mutant is highly sensitive to DNA-damaging agents, such as mitomycin C. The latter agent predominantly causes DNA strand cross-links, leading ultimately to DNA double-strand breaks, which require DNA recombination for repair [14]. We speculated that the 5 patients for whom *H. pylori* was detected in the trabeculum might have had *H. pylori recN* mutants resistant to mitomycin C. In addition, a patchy distribution of *H. pylori* colonization in the stomach has been observed in humans [15], thereby requiring multiple and large-size biopsy specimens to detect the bacteria. If this is also the case of *H. pylori* colonization in the eye tissue, the expected very small size of the tissue samples obtained from POAG patients during trabeculectomy surgery could be a major inhibitor for the detection of *H. pylori* bacteria in the eye. However, further studies are needed to elucidate the aforementioned considerations. It must be emphasized that, although we did not use molecular assays to identify *H. pylori* bacteria in the eye tissues, it is quite unlikely that the observed bacterial populations of eye tissue

surfaces were the result of potential contamination of the solutions used for staining or tissue processing for the following reasons: (1) with the exception of *H. pylori* bacteria identified in the trabeculum and iris of the 5 patients, we did not identify bacteria or bacteria-like populations on the eye tissue surfaces in all the other 46 POAG patients, thereby excluding the aforementioned potential contamination; (2) in routine practice such contamination of the solutions used for staining or tissue processing is extremely rare or even nonexistent; (3) the bacteria stained with cresyl violet exhibited typical morphological features thereby establishing the presence of *H. pylori* bacteria; (4) specifically, cresyl violet staining is used to identify *H. pylori* or *Campylobacter*-like organisms which have the previously mentioned strong affinity for this stain, even very small numbers can be identified with confidence [13] and it excludes other types of bacteria including, for instance, *Staphylococcus aureus*, *Streptococcus pneumoniae* or other possible bacteria which: (a) show quite different morphologic features easily differentiated from the observed typical *H. pylori* bacterial morphology and (b) have been involved in rare cases of postoperative endophthalmitis [16, 17], and (5) all 51 POAG patients did not exhibit symptoms or signs suggesting possible bacteria-related endophthalmitis development following trabeculectomy.

It is important to note that cresyl violet is used not only to detect *H. pylori,* but also in demyelination assessment. Cresyl violet stains both neurons and glia and has been used to stain retinal ganglion cells [18]. However, the tissue samples we obtained did not consist of neural tissue, but of ophthalmic tissue, i.e. trabeculum and iris. Furthermore, *H. pylori* bacteria were stained on the surface of the tissue obtained and thus they could not have been considered as stained ophthalmic cells.

Our study has relied primarily on gastric mucosa histology for the documentation of *H. pylori* infection including the aforementioned 5 cases. Although culture is the theoretical gold standard for detection of the bacterium, it has been shown that there is an excellent correlation with histologic identification [19]. Therefore, for most studies, mucosal biopsy and histologic examination of the specimen for the presence of *H. pylori* and gastritis are the actual gold standards for diagnosis of *H. pylori* infection [3, 20, 21], whereas gastric mucosa urease testing is insensitive, especially in the elderly [22]. However, an upper GI endoscopy is required to obtain specimens for histology or culture of *H. pylori,* and this method is costly and sometimes uncomfortable for patients and may lead to complications, particularly cardiopulmonary problems, in the elderly. This also includes glaucoma patients, representing a special group of patients, as they usually have a higher incidence of comorbid diseases such as chronic heart or lung disease [23, 24]. Therefore, for screening purposes and/or following up the efficacy of *H. pylori*, noninvasive tests including urea breath tests are widely recommended in primary care settings. The ^{13}C-urea breath test, used in our patients, is considered the noninvasive test of choice for the detection of *H. pylori* infection [25]. Therefore, we introduced this test for our 8 POAG patients who were not eligible or who refused to undergo an endoscopy. Although the serological test establishes the presence of antecedent *H. pylori* infection, it does not discriminate between current and old infections. Such a distinction is crucial because only current *H. pylori* infection induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with host epitopes, thereby contributing and possibly perpetuating the glaucomatous tissue damage [26]. Moreover, eradicating *H. pylori* infection might delay glaucoma progression, particularly at early stages of the disease.

Initially, we had proposed the following pathological mechanisms by which *H. pylori* might elicit glaucomatous neuropathy: (1) promoting platelet and platelet leukocyte aggregation; (2) releasing proinflammatory and vasoactive substances, such as cytokines (interleukins 1, 6, 8, 10, 12, tumor necrosis factor-α, interferon-γ), eicosanoids (leukotrienes, prostaglandins), and acute phase proteins (fibrinogen, C-reactive protein) involved in various vascular disorders (migraine, systemic hypertension, Raynaud's phenomenon, cardiovascular disease, and possibly glaucoma) [21]; (3) stimulating mononuclear cells to induce a tissue factor-like procoagulant activity that converts fibrinogen into fibrin; (4) causing the development of cross-mimicry between endothelial and *H. pylori* antigens; (5) producing oxidative stress and circulating lipid peroxides [27], and (6) influencing the apoptotic process. These variables might also exert their own effects in the induction or progression of glaucomatous neuropathy [28] and other neurodegenerative disorders (Guillain-Barré syndrome, Parkinson's disease, Alzheimer's disease, AD) associated with both *H. pylori* infection and glaucoma [21].

Regarding AD, which shares similar risk factors and pathogenetic mechanisms with glaucoma [3, 26, 29], an association between *H. pylori* infection and AD-/mild cognitive impairment-suffering Greek patients has also been found. *H. pylori* eradication may positively influence AD manifestations, and *H. pylori*-specific IgG anti-

body levels are significantly increased in the cerebrospinal fluid of AD patients. Its concentration in cerebrospinal fluid might reflect the AD severity, thereby supporting a role for this common infection in the pathobiology of the disease [30–33]. In this respect, blood-brain barrier (BBB) disruption could play an important role in promoting the entry of immune cell infiltration and pathogens into the brain resulting in the development of brain pathologies [28]. Apart from pathogens' intranasal inoculation, the influx of activated monocytes infected with *Chlamydia pneumoniae* through the BBB could have dire consequences in the brain leading to the development of degenerative diseases, including AD [28], and possibly glaucoma. Infectants including Herpes simplex virus 1, *C. pneumoniae* and even *Borrelia* species have been found in brain regions demonstrating significant AD pathology. Furthermore, the GI tract appears to be a vulnerable area through which pathogens (such as *H. pylori*) may influence the brain and induce neurodegenerative pathologies, mainly via the fast axonal transport by the afferent neurons connecting the GI tract to the brain [34].

Comparable data may also be introduced for *H. pylori*-induced glaucomatous pathologies, although it is not clear how *H. pylori* can reach the trabeculum and iris. This bacterium has been found in the nasal cavity [35] and mouth [36], and the oral cavity might act as a permanent reservoir for this bacterium [37]. Recent studies have shown that from the oral cavity, *H. pylori* may reach the anterior surface of the eye through the nasal cavity, causing blepharitis or worsening it, at least on the basis of cytological criteria [38]. Therefore, as in the case of AD, *H. pylori*'s intranasal route appears to explain, at least partly, the demonstrated presence of *H. pylori* in a few tissue samples obtained during trabeculectomy, thereby having a major impact on understanding the role of this bacterium in the pathogenesis of POAG.

Moreover, specific antibodies are found in increased levels in glaucoma patients' sera, and when these antibodies access the brain due to BBB disruption, they are capable of killing retinal cells, thereby contributing to glaucoma pathologies [26]. Specifically, comparable data could also be considered in the presence of blood-ocular barrier dysfunction. In this respect, a series of factors have been implicated in inducing BBB disruption, including inflammatory mediators (e.g. cytokines and chemokines induced by *H. pylori* infection) and oxidative stress [26]. *H. pylori* could indirectly affect the brain and other target organs, such as the heart or eye through the release of numerous cytokines such as tumor necrosis factor-α acting at a distance. Tumor necrosis factor-α is involved in BBB disruption through a mechanism involving matrix metalloproteinases' upregulation [26]. In addition, *H. pylori*-induced cytotoxin *VacA* exhibits chemotactic activities to the bone marrow-derived mast cells, and mast cells can be stimulated by a corticotropin-releasing hormone, secreted under stress, to release mediators including histamine, interleukin 8, tryptase and vascular endothelial growth factor, which disrupt the BBB [26]. In this regard, as in the case of an influx of activated monocytes infected with *C. pneumoniae* through the BBB, activated monocytes (possibly infected with *H. pylori* due to defective autophagy resulting in *H. pylori* replication in autophagic vesicles [39]) or *H. pylori* antibodies circulating in the bloodstream might also enter the aqueous circulation due to BBB/blood-ocular barrier disruption, possibly contributing to glaucoma development and progression [6]; the *H. pylori VacA* promotes intracellular survival of the bacterium and modulates host immune responses [40].

The main objective of this study was to show that *H. pylori* bacteria do exist locally in incisional biopsies from the trabeculum and iris in POAG patients, and indeed *H. pylori* bacteria were locally demonstrated for the first time. The study did not aim to prove an increased prevalence of *H. pylori* bacteria in POAG patients versus a control group based on biopsy specimens from the eye. Such an objective would be rather unethical, firstly because it would require surgically obtaining biopsy specimens from the trabeculum and iris of healthy patients or from patients who undergo another type of eye operation, and secondly because obtaining a biopsy specimen even from the eyes of *H. pylori*-infected POAG patients can lead to false-negative results for the reasons analyzed previously, thereby leading to an unreliable comparison.

In conclusion, this study has shown for the first time the detection of *H. pylori* bacteria in the trabeculum and iris of POAG patients, indicating that the bacterium is present locally and is possibly directly implicated in glaucomatous damage. The proposed *H. pylori* mechanisms involved in glaucomatous pathologies indeed merit future investigation.

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demographics in REM sleep behavior disorder [2], and therefore could not be included in the discussion. Their prior findings of high rates of RBD symptoms among psychiatric patients taking serotonergic antidepressants [3] concur with the findings in our case series [2] and other reports [4], and we read with interest their follow-up study with polysomnographic confirmation, which is critical for distinguishing RBD from its multiple mimics. The intriguing finding that, despite a decrease in subjective dream enactment episodes with antidepressant change or discontinuation, there was persistent REM without atonia (RWA) noted objectively on polysomnogram [1], lends further credence to a contributory role of antidepressants in RBD and RWA in susceptible individuals. However, studies with polysomnograms before and after antidepressant initiation, demonstrating RBD occurring as a consequence of antidepressants, are still lacking. We certainly agree that further investigation is necessary in this area, particularly as antidepressant usage becomes increasingly widespread. Additionally, long-term follow-up studies are required to determine any relationship between antidepressant-related RBD and neurodegenerative disease.

We further agree that our finding of a relationship between autoimmune disorders and RBD may be confounded by antidepressant usage in this group. As we noted in our paper, "Referral bias may have contributed to the high rates of autoimmune disease in this series, since patients with autoimmune disease may have fatigue, pain causing poor sleep, and other reasons to be referred to sleep medicine. Moreover, antidepressant usage, which has been associated with RBD, may be relatively higher in those with autoimmune disease." [2]. Indeed, four of our eight subjects with autoimmune diseases were also taking antidepressants. However, there are clear neuro-autoimmune diseases which have been reported to manifest with RBD, including another recent report of anti-Ma antibody associated RBD and cataplexy [5], in addition to those already discussed in our paper [6,7]. Due to the small population of patients with autoimmune disorders, the widespread use of antidepressants in this population, and the overall rarity of RBD, a larger study, likely with cooperation from multiple centers focusing on recruitment from rheumatology or neuro-immunology clinic populations, would be required to tease apart this possible confound.

Conflict of interest

The ICMJE Uniform Disclosure Form for Potential Conflict of Interest associated with this article can be viewed by clicking on the following link: doi:10.1016/j.sleep.2011.05.001.

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Helicobacter pylori may play a role in both obstructive sleep apnea and metabolic syndrome

To the Editor

We read with interest the paper by Theorell-Haglöw et al. [1], which concluded that obstructive sleep apnea (OSA) is closely associated with metabolic syndrome and its components in a Swedish population.

Regarding a relative, though still debatable, issue, *Helicobacter pylori* infection (*Hp*-I) may be involved in the pathogenesis of both OSA and gastroesophageal reflux disease (GERD); the latter disorder is also associated with OSA [2,3]. Based on the histological analysis of gastric mucosa biopsy for *Hp*-I documentation, we reported an association between *Hp*-I and GERD and non-endoscopical reflux disease [2]. Moreover, *Hp* eradication leads to better control of GERD symptoms and improves esophagitis [2]; GERD is significantly associated with OSA independent of body mass index and the occurrence of GER after bedtime is strongly associated with OSA symptoms [3]. In an additional pilot study we also reported an association between *Hp*-I and OSA [4]. Moreover, increased levels of inflammatory mediators, including tumor necrosis factor-α, interleukin (IL)-1β, IL-8 and reactive oxygen species, were observed in 46.6%, 46.6%, 53.3%, and 60.0% of our patients, respectively [4]. It is important to note that these inflammatory mediators are involved in OSA pathophysiology; OSA is linked to increased oxidative stress, decreased antioxidant defense, and systemic inflammation, which characterizes OSA patients.

Finally, in a recent review [5], we reported a potential association between *Hp*-I and insulin resistance (IR), though further studies are needed to strengthen this association. In this regard, OSA is an independent risk factor for IR, as mentioned by the authors, and IR represents the pathogenetic basis of metabolic syndrome.

Viewing the aforementioned data, and because about half of the world's population is estimated to be infected with *Hp*, it would be interesting to know if the authors have considered *Hp*-I as a potential con-founder involved in OSA and GERD pathophysiology associated with metabolic syndrome.

Conflicts of interest

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What is cause and what is effect?

From the authors:

We would like to thank Prof. Kountouras and co-workers for their interest in our article [1]. Kountouras et al. raise the question whether *Helicobacter pylori* infection (*Hp*-I) could be a potential confounder involved in obstructive sleep apnea (OSA) and gastro-esophageal reflux disease (GERD) pathophysiology associated with metabolic syndrome. The concept is interesting as OSA, GERD and metabolic syndrome are all common disorders.

The increase in negative intrathoracic pressure during an apnea could potentially give rise to GERD and treatment of OSA has been shown to reduce GERD [2]. Although the role of night-time reflux in connection with apneas is well described in the literature, it is based on studies with poor levels of evidence [3].

As Kountouras et al. states, it is debatable whether *Hp*-I is involved in the pathogenesis of OSA and GERD and, also, metabolic syndrome. Both GERD and OSA are common and it is therefore also possible that we are simply looking at two common conditions that may well coexist in an individual. In addition, as both GERD and OSA are associated with obesity, this may affect the relationship with metabolic syndrome. Our study in women was cross-sectional and it is therefore difficult to say what is cause and effect. One can only speculate as to the role of *Hp*-I in the association between OSA and metabolic syndrome. Furthermore, the prevalence of *H. pylori* in Uppsala is low [4], and the possibility to use *Hp*-I as a confounder is limited in the current population.

Conflicts of interest

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Desaturation during sleep in mild to moderate COPD: A marker of poor sleep quality alone?

To the Editor,

In their interesting and well conducted study on sleep profiles and symptoms of sleep disorders in COPD patients, Valipour et al. found new evidence that nocturnal desaturation is significantly linked to symptoms in independent scales of the questionnaire on sleep disorders in mild to moderate stages of COPD [1].

A study conducted in our group of patients with mild to moderate COPD (GOLD) [2], comparing the T90 (percentage of sleep time with hemoglobin saturation under 90%) of desaturators (T90 of 12.3%) and non-desaturators (T90 of 0.2%, $p < 0.0001$), showed no difference in the sleep architecture between the groups (unpublished data), but there was a significant increase in hs-CRP (high-sensivity C reactive protein) in the desaturator group ($p < 0.05$).

Our study also showed a high incidence of OSA, with 54% of eligible patients of our study population having an AHI >10/h. Both studies indicate it would take a more marked degree of nocturnal desaturation to produce sleep arousals, but light nocturnal desaturation could take longer to trigger slow-grade systemic inflammation and poorer sleep quality.

Clinical Ophthalmology

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REVIEW

Helicobacter pylori infection and primary open-angle glaucoma: is there a connection?

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Abstract: Glaucoma, the most common form of which is primary open-angle glaucoma (POAG), is a neurodegenerative disease which is the second most common cause of blindness worldwide. Among the factors that have been implicated in the pathophysiology of this disease is infection with *Helicobacter pylori*. This alleged association has caused a great deal of scientific discussion during the past decade, as the establishment of such a correlation might lead to therapeutic applications for all glaucoma patients. In this review, we assess all relevant major studies and trials in an effort to elucidate the issue.

Keywords: *Helicobacter pylori*, primary open-angle glaucoma, apoptosis

Introduction

Glaucoma is a neurodegenerative disease which is the second most common cause of blindness worldwide. It is estimated that 60 million people in the world are affected by the disease and 8.4 million are bilaterally blind.[1] Various factors, among them infection with *Helicobacter pylori* (HP), a Gram-negative bacterium associated with the occurrence of Alzheimer's disease, gastritis, gastric ulcers, and gastric carcinomas,[2–4] have been implicated in the pathophysiology of the disease. Over the last decade, researchers around the world have focused on the issue; however, the controversial results of studies have left the subject open to further discussion. Another question that will require answers if an association between HP infection and glaucoma is proven, is whether these two entities are linked by a causal relationship or whether they share common predisposing or precipitating factors.

Studies

Kountouras et al[5] were among the first to investigate the putative association between HP infection and glaucoma. In a prospective study (based on a sample of 32 patients with primary open-angle glaucoma (POAG), nine patients with pseudoexfoliation glaucoma (PEG) and a control group of 30 age-matched anemic patients), they concluded that HP infection is more frequently encountered among glaucoma patients than in the general population; this finding was based on both biopsy-based campylobacter-like organism tests as well as on serological examinations. Glaucoma patients also had, to a statistically significant extent, more frequent pathologies in the form of antral gastritis and peptic ulcers. In an extension of this study,[6] the same authors proved that in the long term, glaucoma patients benefit in terms of regulation of intraocular pressure and visual field parameters in those cases where HP is successfully eradicated. It was again the

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same team[7] who conducted a prospective study on 26 POAG patients and 27 PEG patients undergoing glaucoma surgery and 31 patients undergoing phacoemulsification cataract surgery and concluded there was an increased concentration of IgG HP antibodies in the aqueous humor of surgical eye specimens from glaucoma patients. These studies by Kountouras et al were used in correspondence[8] addressed to Weinreb et al,[9] in which Kountouras criticized the lack of mentioning of the role of HP in glaucoma by Weinreb.

Deshpande et al[10] also performed a similar prospective, nonrandomized comparative study based on the serological and aqueous humor examination of 50 glaucoma patients and a control group of patients undergoing phacoemulsification cataract surgery. They found a statistically significant difference between POAG patients and PEG and control patients, as far as the concentration of serum HP IgG antibodies was concerned. However, they did not find any significant correlations between the patient groups in regard to HP IgG antibody concentration in the aqueous humor. Galloway et al[11] performed a prospective study based on a sample of 97 patients with POAG, PEG, and ocular hypertension and an analogous control group; they did not find statistically significant differences in the concentration of serum HP-IgG antibodies between groups, thus leaving an open field for further research and scientific discussion. Similarly, Kurtz and associates[12] investigated seropositivity to HP and to its cytotoxin-associated gene A (CagA) product in 51 patients with various types of glaucoma and compared the findings to those of a control group of 36 cataract patients. They found no significant association between HP infection and CagA seropositivity with glaucoma. Handa et al noted the connection of CagA to various pathological pathways, leading to irregular gastric cell activity, apoptosis, and carcinogenesis.[13]

There has also been much debate regarding the mechanisms that might be involved in the possible association of HP infection with glaucoma. Is there a causal relationship between them or do they share common predisposing or precipitating factors and pathophysiological backgrounds? Various authors[14–19] believe that HP infection acts through the release of proinflammatory and vasoactive substances, through the release of endothelin-1, nitric oxide, and oxygen free radicals, and through the induction of oxidative stress, the destruction of mitochondrial DNA and the induction of apoptotic cell death, which may be the main reason for glaucomatic neuropathy. This apoptotic process may be linked with the FASL protein, a type-II transmembrane protein belonging to the tumor necrosis factor (TNF) family. The binding of this protein with its receptors differentiates the response of the immune system, is believed to play a role in oncogenetic processes, and is also connected with apoptotic cell death.[14,15,18]

Discussion

Over the last decade, various researchers have examined the existence of a possible link between HP infection and glaucoma. Some studies have supported such an association, with suggested underlying mechanisms including the induction of inflammatory responses as well as apoptotic processes that lead to glaucomatic neuropathy. Although some support for this seems to exist, contradictory results from other related studies leave room for further investigation. This controversy is best reflected in the scientific debate that took place between Weinreb et al[9] and Kountouras et al,[8] with Kontouras et al claiming there was a positive correlation between HP and glaucoma. The establishment of such a causal relationship will probably have important practical applications as the eradication of HP might lead to developments in the treatment of glaucoma.

Disclosure

The authors report no conflicts of interest in this work.

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A potential impact of chronic *Helicobacter pylori* infection on Alzheimer's disease pathobiology and course

In their case-control series, Roubaud-Baudron et al. concluded that Alzheimer's disease (AD) patients infected by *Helicobacter pylori* (*Hp*) tended to be more cognitively impaired, though studies are needed to attest to the impact of *Hp* infection (*Hp*-I) on AD course, especially on cerebrovascular lesions and neuroinflammation (Roubaud-Baudron et al., 2011). We herein wish to add our own data regarding mainly the impact of *Hp* eradication on AD course; *Hp* eradication potentially, by reducing AD severity and/or altering its pathophysiology, might lead to prolonged survival of AD patients, thereby having an impact on the natural history of this disease.

Estimates of median survival from the onset of symptoms of dementia revealed 3.1 years for patients with probable AD, 3.5 years for patients with possible AD, and 3.3 years for patients with vascular dementia (Wolfson et al., 2001). We tested the hypothesis that eradication of *Hp*-I might improve the survival rate, thereby decreasing mortality in a Greek cohort of AD patients (Kountouras et al., 2010). During a 5-year follow up (47.19 ± 15.11 months), overall 21 (of 46 patients eventually enrolled in our analysis) died and 25 patients remained alive. Patients who died were older and exhibited lower mean Mini Mental State Examination (MMSE) score compared with the patients still alive. Successful eradication of *Hp*-I was associated with a significantly lower mortality risk (hazard ratio [95% confidence interval]: 0.287 [0.114–0.725]; $p = 0.008$). The results were similar in adjusted and unadjusted models, for age and Mini Mental State Examination score at baseline. We concluded that *Hp* eradication regimen in AD patients is associated with a higher 5-year survival rate, thereby possibly having an impact on AD course.

Hp-I might influence the pathophysiology of AD and other neurodegenerative diseases through several mechanisms, including the release of proinflammatory and vasoactive substances (e.g., cytokines or eicosanoids), induction of oxidative stress, or apoptotic processes (Kountouras, 2009; Kountouras et al., 2007). Specifically, blood-brain barrier (BBB) damage has been increasingly implicated in AD and other inflammatory demyelinating neuropathies and a variety of proinflammatory cytokines, also mentioned by the authors (Roubaud-Baudron et al., 2011), are instrumental in the course of these diseases. These cytokines increase vascular permeability and BBB disruption (tumor necrosis factor [TNF]-alpha, vascular endothelial growth factor [VEGF], vascular permeability factor), they induce transmigration of leukocytes into the nerve, activation, and proliferation of macrophages (interferon [IFN]-gamma), and T cells (interleukin [IL] −1 and −2), and exert direct myelinotoxic activities (TNF-alpha and TNF-beta) (Kountouras et al., 2011).

Specifically, inflammatory mediators (e.g., cytokines and chemokines induced by *Hp*-I) and oxidative stress have been implicated in inducing BBB disruption. *Hp* could indirectly affect the brain through the release of numerous cytokines such as TNF-alpha, acting at a distance; TNF-alpha is involved in BBB disruption through a mechanism involving matrix metalloproteinases upregulation. In addition, *Hp*-induced cytotoxin VacA exhibits chemotactic activities to the bone marrow-derived mast cells (BMDMCs) and induces BMDMCs to produce proinflammatory cytokines which disturb the BBB; BMDMCs reside adjacent to blood and lymphatic channels, mainly under epithelial surfaces including the BBB and gastrointestinal tract (Kountouras, 2009; Kountouras et al., 2011). Apart from activated mast cells, VEGF, IL-8, chymase, or tryptase and mast cell growth factor linked to *Hp*-I, mast cells themselves can be stimulated by corticotropin-releasing hormone, secreted under stress, to release mediators including histamine, IL-8, tryptase, and VEGF, which disrupt the BBB (Kountouras, 2009; Kountouras et al., 2011); mast cell degranulation is able to secrete these potent mediators which could orchestrate neuroinflammation and affect the BBB integrity, thereby playing a role in neuropathies. The "cross-talk" between mast cells, lymphocytes, neurons, and glia constitutes a neuroimmune axis implicated in a range of neurodegenerative diseases with an inflammatory and/or autoimmune component including AD (Kountouras et al., 2011). In turn, BBB disruption could play an important role in promoting entry of immune cell (autoreactive effector $CD4^+$ and $CD8^+$ T cells) infiltrations and pathogens into the brain resulting in the brain pathologies; apart from the pathogens' entry into the brain through the nasal/olfactory pathway, BBB breakdown promotes the entry of activated monocytes infected with pathogens, also mentioned by the

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authors (Roubaud-Baudron et al., 2012), into the brain, leading to a significant AD pathology. A similar entry of activated monocytes infected with *Hp*, due to defective autophagy resulting in *Hp* replication in autophagic vesicles and/or *Hp* circulating antibodies might also lead to the development of neuronal pathologies possibly including AD (Kountouras et al., 2011).

Furthermore, *Hp*-n, a histidine-rich protein abundant in this bacterium and which forms amyloid-like oligomers, could be transported through the BBB by the main low-density lipoprotein receptor-related protein-1 (LRP-1) and receptor for advanced glycation end products (RAGE) transporters for amyloid-β-peptide (Aβ) across the BBB from brain to blood and blood to brain, thereby playing a role in AD pathophysiology; *Hp*-I, by releasing the above-mentioned inflammatory mediators leads to BBB disruption, which, in turn, apart from LRP-1 and RAGE, might promote free entry of *Hp*-n proteins into the brain resulting in the development of AD pathologies by inducing Aβ fibril formation or aggregating synergistically with Aβ (Zavos et al., 2012).

Summarizing the aforementioned data, *Hp*-I may be involved in BBB disruption leading to cerebrovascular lesions and neuroinflammation, so that eradicating *Hp*-I may have a major impact on AD course. However, further studies are needed to elucidate this field.

Disclosure statement

The authors disclose no conflicts of interest.

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Potential implications of *Helicobacter pylori*-related neutrophil-activating protein

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Abstract

Helicobacter pylori (*H. pylori*) virulence factors promote the release of various chemoattractants/inflammatory mediators, including mainly the neutrophil-attractant chemokine interleukin-8 and neutrophil-activating protein (NAP), involved in *H. pylori*-induced gastric pathologies. Co-administration of Chios mastic gum (CMG), which inhibits *H. pylori* NAP, with an *H. pylori* eradication regimen might add clinical benefits against *H. pylori*-related gastric pathologies, but possibly not CMG as main therapy. Although *H. pylori* NAP and other *H. pylori*-related cytotoxins [i.e., vaculating cytotoxin (VacA)] appear to play a major role in generating and maintaining the *H. pylori*-associated gastric inflammatory response and *H. pylori* NAP is a promising vaccine candidate against *H. pylori* infection (*H. pylori*-I), concerns regarding its potential drawbacks, particularly neurogenic ones, due to possible cross-mimicry, should be considered. Possible cross-mimicry between *H. pylori* NAP and/or bacterial aquaporin (AQP) and neural tissues may be associated with the anti-AQP-4 antibody-related neural damage in multiple sclerosis (MS)/neuromyelitis optica patients. Moreover, the sequence homology found between *H. pylori* VacA and human Na+/K+-ATPase A subunit suggests that antibodies to VacA involve ion channels in abaxonal Schwann cell plasmalemma resulting in demyelination in some patients. A series of factors have been implicated in inducing blood-brain barrier (BBB) disruption, including inflammatory mediators (e.g., cytokines and chemokines induced by *H. pylori*-I) and oxidative stress. BBB disruption permits access of AQP4-specific antibodies and T lymphocytes to the central nervous system, thereby playing a major role in multiple sclerosis pathogenesis. Relative studies show a strong association between *H. pylori*-I and MS. *H. pylori*-I induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves, thereby contributing and perpetuating neural tissue damage. Finally, *H. pylori* NAP also plays a possible pathogenetic role in both gastric and colon oncogenesis.

Key words: *Helicobacter pylori*; Neutrophil-activating protein; Chios mastic gum; Cross-mimicry; Multiple sclerosis; Demyelination; Gastric carcinogenesis

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TO THE EDITOR

In their recent paper published in this journal, Choli-Papadopoulou *et al*[1] consider the development of new drugs targeting *Helicobacter pylori* (*H. pylori*) neutrophil-activating protein (NAP) and this raises some concerns.

With reference to a study[2] focusing on *H. pylori* NAP-mediated neutrophil activation before and 2 mo after *per os* administration of Chios mastic gum (CMG), the authors claimed that "these results indicate a substantial down-regulation of the innate cellular immune effectors, which, according to unpublished clinical data in the context of this study, are accompanied by a significant clinical improvement of the patients' complaints (dyspepsia, epigastric discomfort, distention)"[1]. However, such clinical benefits cannot be deduced from this study[2] and, as mentioned, relative clinical data on CMG as treatment for *H. pylori* and peptic ulcer are controversial[2]. Although *H. pylori* virulence factors promote the release of various chemoattractants/inflammatory mediators including mainly the neutrophil-attractant chemokine interleukin-8 and *H. pylori* NAP involved in *H. pylori*-induced gastric pathologies[3], our clinical experience suggests that only co-administration of CMG with an *H. pylori* eradication regimen might add clinical benefits against *H. pylori*-related gastric pathologies, but possibly not CMG as main therapy, as the authors claimed[1,2]. In particular, co-administration of CMG might be a potential therapy to reduce damage of gastric mucosa induced by *H. pylori* NAP. However, large-scale relative prospective studies are needed to elucidate this field.

The authors, further considering data on the safety and immunogenicity of a vaccine comprising *H. pylori*-induced vaculating cytotoxin (VacA), cytotoxin associated gene and *H. pylori* NAP, suggested that the obtained neutrophil activation by the C-terminal region of *H. pylori* NAP opens new pathways for drug design directed at *H. pylori* inflammation[1]. In particular, both VacA and *H. pylori* NAP play a major role in generating and maintaining the *H. pylori*-associated gastric inflammatory response, and *H. pylori* NAP is a promising vaccine candidate against *H. pylori* infection (*H. pylori*-I). However, concerns regarding potential drawbacks of *H. pylori* NAP, particularly neurogenic ones, should be considered. For instance, possible cross-mimicry between *H. pylori* NAP and/or bacterial aquaporin (AQP) and neural tissues may be associated with the anti-AQP-4 antibody-related neural damage in multiple sclerosis (MS)/neuromyelitis optica (NMO) patients. In this regard, by using histology, the practical gold standard for the diagnosis of *H. pylori*-I, we have shown a strong association between *H. pylori*-I and MS[4]. *H. pylori*-I induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves, thereby contributing and perpetuating neural tissue damage[4]. In this respect, *H. pylori* NAP, as a virulence factor, recruits leukocytes from the vascular lumen, and activates neutrophils, monocytes and mast cells, as mentioned by the authors. Besides, the sequence homology found between *H. pylori* VacA and human Na+/K+-ATPase A subunit suggests that antibodies to VacA involve ion channels in abaxonal Schwann cell plasmalemma resulting in demyelination in some patients[5]. Moreover, VacA exhibits chemotactic activities to the bone marrow-derived mast cells (BMDMCs) and induces BMDMCs to produce pro-inflammatory cytokines[5]. A series of factors have been implicated in inducing blood-brain barrier (BBB) disruption, including the aforementioned inflammatory mediators (e.g., cytokines and chemokines induced by *H. pylori*-I) and oxidative stress. BBB disruption permits access of AQP4-specific antibodies and T lymphocytes to the central nervous system, thereby playing a major role in MS/NMO pathogenesis[6]. Therefore, *H. pylori* NAP and *HP*-I itself, by inducing several mediators, may influence MS/NMO (including relapsing type) pathophysiology, thereby raising possible concerns regarding even the C-terminal region of *H. pylori* NAP use as a candidate vaccine. Accordingly, relative studies are also needed to clarify the aforementioned concerns.

Finally, the possible *H. pylori* NAP pathogenetic role in gastric carcinogenesis, mentioned by the authors, may also apply to colon oncogenesis[2,7].

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LETTER

Further data on the association between *Helicobacter pylori* infection and primary open-angle glaucoma

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We herein wish to provide data additional to those reported in our previous review (Tsolaki et al[1]) concerning the association between *Helicobacter pylori (H. pylori)* infection and primary open-angle glaucoma. These data were not available in the time period when the review paper was submitted and accepted for publication, and we believe that they need to be added because they further reinforce this association.

In our new study published this year,[2] we show for the first time the existence of *H. pylori* bacteria in the trabeculum and iris specimens of glaucoma patients, thereby further supporting the role for *H. pylori* infection in the pathophysiology of primary open-angle glaucoma. Specifically, the study included 51 consecutive patients who underwent trabeculectomy for primary open-angle glaucoma not responsive to topical antiglaucoma therapy. The presence of *H. pylori* was established by upper gastrointestinal endoscopy and histology, or by a urea breath test in eight patients who either were deemed not suitable endoscopy candidates or refused to undergo endoscopy.

All the patients underwent a surgical trabeculectomy procedure to their eyes, during which tissue samples from the trabeculum, conjunctiva, and iris were immediately obtained, placed in tubes containing 10% formalin, and submitted for histological examination. These specimens were stained using the Cresyl fast violet method (for detection of *H. pylori* organisms). In five patients, for whom gastric *H. pylori* histology was positive, we managed to identify *H. pylori* bacteria in the trabeculum and iris specimens histologically with Cresyl fast violet stain for the first time. The reason why *H. pylori* was not found in the trabeculum and iris of all primary open-angle glaucoma patients who tested positive for *H. pylori* status could be explained, apart from the absence of *H. pylori* in the eye, by the very small size of the sample of tissue obtained and submitted for histopathology during trabeculectomy, or possibly by standard local antisepsis prior to surgery. Despite the small number of cases, the findings of this study are important because *H. pylori* bacteria have been detected for the first time in the trabeculum and iris of patients with primary open-angle glaucoma, confirming that the bacterium is present locally, and is possibly directly implicated in glaucomatous damage.

In a second study just accepted for publication,[3] we obtained biopsy specimens at upper gastrointestinal endoscopy from 43 patients with primary open-angle glaucoma, which were then evaluated for the presence of *H. pylori*, for expression of genes involved in cell proliferation and apoptosis (Ki-67, p53, Bcl-2), and for indices of cellular immune surveillance (T lymphocytes [TLs] and B lymphocytes [BLs]). Of the

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43 patients eligible for upper gastrointestinal endoscopy, 90.7% tested positive for *H. pylori* infection. Ki-67 was positively expressed in 81.25% of patients with *H. pylori* infection and in one patient without *H. pylori* infection. p53 was positively expressed in 31.25% of patients with *H. pylori* infection but not in those without *H. pylori* infection. Bcl-2 was positively expressed in 68.75% of patients with *H. pylori* infection and in one patient without *H. pylori* infection. Ki-67, p53, and Bcl-2 were overexpressed in 19%, 25%, and 37.5%, respectively, of patients with *H. pylori* infection, but none was overexpressed in the patients without *H. pylori* infection. The TL marker was positively expressed in all patients with *H. pylori* infection and in the one patient without *H. pylori* infection. The BL marker was positively expressed only in one patient with *H. pylori* infection. Therefore, our forthcoming article provides further evidence that the *H. pylori*-induced oncogenes, Ki-67, p53 and Bcl-2, as well as TL markers are involved in cell proliferation and apoptotic pathways, thereby contributing to glaucomatous neuropathy, with oncogenic potential. In this respect, and apart from the apoptotic processes involved in the pathophysiology of glaucomatous neuropathy, recent experimental data indicate that cell proliferation rather than astrocyte hypertrophy characterizes early pressure-induced optic nerve head injury, and the optic nerve head is the principal site of initial axonal injury in glaucoma. Furthermore, at the eye level, Ki-67 has been used as an index of melanoma proliferation and also as an index showing response to treatment with agents that inhibit the proliferation of Tenon's fibroblasts, decreasing excessive scarring after trabeculectomy.[2]

Specifically, we have also very recently provided an overview of the various pathophysiological mechanisms underlying the association between *H. pylori* infection and primary open-angle glaucoma[4] which include: promoting platelet and platelet-leukocyte aggregation, also involved in the pathophysiology of glaucoma; releasing proinflammatory and vasoactive substances, including cytokines (interleukins-1, -6, -8, -10, and -12, tumor necrosis factor alpha [TNF-α], interferon-gamma), eicosanoids (leukotrienes, prostaglandins) and acute-phase proteins (fibrinogen, C-reactive protein) involved in vascular disorders and glaucoma; stimulating mononuclear cells to induce tissue factor-like procoagulant activity that converts fibrinogen into fibrin; causing the development of cross-mimicry between endothelial and *H. pylori* antigens; producing oxidative stress and circulating lipid peroxides; and in particular influencing the apoptotic process, parameters of which may also exert their own effects in the induction and/or progression of glaucoma and other neurodegenerative disorders (Guillain–Barré syndrome, Alzheimer's disease, Parkinson's disease) associated with both *H. pylori* infection and glaucoma.

Importantly, *H. pylori* infection and glaucoma share the Fas/FasL and mitochondria-mediated apoptotic pathways, thereby suggesting an apoptotic link in the pathophysiology of both diseases. In particular, increased endothelin-1 (a potent constrictor of arterioles and venules), nitric oxide, and inducible nitric oxide synthase levels are associated with *H. pylori* infection. Endothelin-1-induced anterior optic nerve vessel vasoconstriction and vascular tone modulation by nitric oxide in the ophthalmic artery may produce glaucomatous damage. Moreover, nitric oxide, a rapidly diffusing gas, is a potent neurotoxin that may facilitate apoptotic retinal ganglion cell death in glaucomatous optic neuropathy. Support for the consideration of nitric oxide neurotoxicity in glaucoma is provided by experimental evidence demonstrating that retinal ganglion cell apoptosis is attenuated by neutralizing antibodies against TNF-α or by selective inhibitors of inducible nitric oxide synthase, thereby suggesting that the inhibitors of TNF-α or of the inducible isoform (NOS-2) may provide novel therapeutic targets for neuroprotection in the treatment of glaucomatous optic neuropathy.

In addition, systemic *H. pylori*-induced oxidative damage may be the mechanism which links oxidative stress, *H. pylori* infection, and the apoptotic damage to the trabecular meshwork and optical nerve head that results in glaucoma. In this regard, oxidative stress is an essential underlying cause of neuroinflammatory and neurodegenerative diseases, including glaucoma and the blood–brain barrier damage connected to these diseases; oxidative stress activates protein tyrosine kinase and matrix metalloproteinases, resulting in blood–brain barrier dysfunction.[4]

H. pylori infection, by releasing several inflammatory mediators,[5] could induce breakdown of the blood–brain/blood–ocular barriers, thereby being involved in the pathogenesis of neuropathies, including glaucoma.[5] For instance, *H. pylori* could indirectly affect the brain through the release of TNF-α acting at a distance; TNF-α is involved in blood–brain barrier disruption through upregulation of matrix metalloproteinases. Furthermore, circulating antibodies for *H. pylori* might also enter the aqueous circulation due to disruption of the blood–brain/blood–ocular barriers, possibly contributing to the pathophysiology of glaucoma; when serum-specific antibodies access the brain, they are capable of killing retinal cells.[5] Likewise, an influx of *H. pylori*-infected monocytes, owing to defective autophagy

resulting in *H. pylori* replication in autophagic vesicles, through the disrupted blood–brain/blood–ocular barrier, might lead to glaucoma neuropathy. *H. pylori* VacA cytotoxin promotes intracellular survival of the bacterium and modulates host immune responses.[5] In addition, because the oral cavity might act as a permanent reservoir for *H. pylori*, this bacterium may reach the eye through the nasal cavity, causing ophthalmic pathologies, possibly including glaucoma.[5]

Finally, it is important to note that studies conducted in Korea, China, India, Turkey, and Iran have also reported an association between *H. pylori* infection and glaucoma.[2]

Disclosure

The authors report no conflicts of interest in this work.

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LETTER TO THE EDITOR

Helicobacter pylori infection and Parkinson's disease: apoptosis as an underlying common contributor

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Nielsen *et al.* [1] state that *Helicobacter pylori* infection (*Hp*-I) may play an important role in the pathogenesis of Parkinson's disease (PD) by triggering microglia activation through the humeral or vagal afferent pathways.

In this regard, apoptotic rather than necrotic microglia-associated nerve cell death appears to underlie a number of common neurological conditions including PD, Alzheimer's disease (AD), glaucoma or multiple sclerosis [2]. The latter diseases are also associated with *Hp*-I [2–4]; likewise, an association of glaucoma with neurodegenerative diseases (PD and AD) via apoptotic cell death has been reported.

Hp, apart from Fas–FasL apoptotic pathway [2], is capable of inducing apoptotic effects through the mitochondrial apoptotic pathway involving the activation of the pro-apoptotic proteins Bax and Bak, activation of certain caspases, or through inducible nitric oxide (NO); NO is a rapidly diffusing gas and a potent neurotoxin that may contribute to apoptotic neuronal cell death in degenerative neuropathies [4], including PD. In particular, increased endothelin-1 (a potent constrictor of arterioles and venules), NO and inducible nitric oxide synthase (iNOS) levels are associated with *Hp*-I [2]. Relative data in AD indicate that endothelin-1-like immunoreactivity in the AD brains is significantly more increased in frontal and occipital cortex than that in the control brains, thereby explaining the decreased cerebral blood flow in patients with AD. Besides, the synthesis of NO, the peroxynitrite reactive production and the protein tyrosine nitration are activated over the entire chronic course of AD, and the presence of Abeta increases the presence of neuronal NOS and iNOS isoforms over the chronic course of AD in pyramidal-like neurons [2]; NO contributes to mitochondrial fragmentation via S-nitrosylation of dynamin-related protein 1, a protein involved in mitochondrial fission. These findings might provide a new target for drug development in AD. Moreover, redox reactions triggered by excessive levels of NO can contribute to protein misfolding, the hallmark of a number of neurodegenerative disorders, including PD and AD [5].

Finally, apart from humeral and vagal afferent pathways, mentioned by the authors [1], *Hp* might access brain via oral-nasal-olfactory pathway or by circulating monocytes (possibly infected with *Hp* owing to defective autophagy) through disrupted blood–brain barrier, leading to neurodegeneration [3].

Disclosure of conflict of interest

The authors declare no financial or other conflict of interests.

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Correspondence

Glaucoma and *Helicobacter pylori*: Eyes "wide open"!

Sir,

We read with interest Zullo et al.'s Editorial [1] discussing *Helicobacter pylori* infection (*Hp*-I) prevalence, pathogenetic mechanisms and clinical relevance in glaucoma.

Apart from the studies mentioned by the authors [1], there are additional published data further reinforcing the association between glaucoma and *Hp*-I; reports from other ethnic populations [2,3] showed a relationship between glaucoma and *Hp*-I, although not confirmed by all the relevant studies published so far (Table 1), and thus this association applies to ethnic sub-populations of glaucoma patients. Consistent associations with the Greek data were shown in studies from China, Korea, India, Turkey, and Iran [4].

For instance, studies from China showed an association between *Hp*-I and primary open-angle glaucoma (POAG) by using ^{13}C-urea breath test, the broadly used non-invasive diagnostic test of choice, having a significantly higher diagnostic accuracy than serology. Moreover, studies from Turkey and Iran, also reported a comparable association by using serology, aqueous humour anti-*Hp* IgG antibodies and/or histology.

Hp-I may influence the pathophysiology, and therefore the management of glaucoma, by the following several mechanisms [3–5], including those reported by the authors [1]: (1) promoting platelet and platelet leucocyte aggregation; (2) releasing proinflammatory and vasoactive substances, such as cytokines (interleukins 1, 6, 8, 10, 12, tumour necrosis factor (TNF)-α, interferon-γ), eicosanoids (leukotrienes, prostaglandins), and acute phase proteins (fibrinogen, C-reactive protein) involved in various vascular disorders and possibly glaucoma; (3) stimulating mononuclear cells to induce a tissue factor-like procoagulant activity that converts fibrinogen into fibrin; (4) causing the development of cross mimicry between endothelial and *Hp* antigens; (5) producing oxidative stress and circulating lipid peroxides; and (6) influencing the apoptotic process.

Table 1
Studies from diverse ethnic glaucoma populations investigating a relationship between *Helicobacter pylori* infection and glaucoma. Negative indicates lack of association.

Year published	Authors	Country	Outcome
2000	Kountouras et al.	Greece	Positive
2000	Öztürk et al.	Turkey	Positive
2001	Kountouras et al.	Greece	Positive
2002	Kountouras et al.	Greece	Positive
2003	Kountouras et al.	Greece	Positive
2003	Galloway et al.	Canada	Negative
2003	Jahadi et al.	Iran	Negative
2004	Roozitalab et al.	Iran	Positive
2005	Quaranta et al.	Italy	Negative
2006	Razeghinejad et al.	Iran	Positive
2007	Abrishami et al.	Iran	Positive
2007	Hong et al.	China	Positive
2008	Kurtz et al.	Israel	Negative
2008	Deshpande et al.	India	Positive
2009	Öztürk et al.	Turkey	Positive
2011	Kim et al.	Korea	Positive
2012	Zavos et al.	Greece	Positive

Specifically, *Hp*-I, by releasing several inflammatory mediators, could induce blood–brain barrier (BBB)/blood–ocular barrier (BOB) breakdown, thereby being involved in the pathogenesis of neuropathies including glaucoma [2–4]. For instance, *Hp* could indirectly affect the brain through the release TNF-α acting at a distance; TNF-α is involved in BBB disruption through matrix metalloproteinases' upregulation. Furthermore, *Hp* circulating antibodies might also enter the aqueous circulation due to BBB/BOB disruption possibly contributing to glaucoma pathophysiology; when serum-specific antibodies access the brain, they are capable of killing retinal cells. Likewise, an influx of *Hp*-infected monocytes, owing to defective autophagy resulting in *Hp* replication in autophagic vesicles, through the disrupted BBB/BOB might lead to glaucoma neuropathy. *Hp* VacA cytotoxin promotes intracellular survival of the bacterium and modulates host immune responses; *Hp* VacA also exhibits chemotactic activities to the bone marrow-derived mast cells, and mast cells can be stimulated by corticotropin-releasing hormone, secreted under stress, to release mediators including histamine, interleukin 8, tryptase and vascular endothelial growth factor, which disrupt the BBB/BOB. Finally, since the oral cavity might act as *Hp* permanent reservoir, this bacterium may reach the eye through the nasal cavity, causing ophthalmic pathologies possibly including glaucoma. Therefore, *Hp*'s intranasal route appears to explain, at least partly, the demonstrated presence of *Hp* in a few tissue samples obtained during trabeculectomy in our series, thereby having a major impact on understanding the role of this bacterium in the pathogenesis of POAG. Unfortunately, due to the very small size of the sample of tissue obtained and submitted for histopathology during the trabeculectomy, the bacteria were identified only by using Cresyl fast violet specific stain whereas no sufficient residue tissue specimens remained for immunological assessment as mentioned by the authors [1]. Having obtained large size samples from other tissues, for instance colon neoplasm tissues, we documented *Hp* presence both by Cresyl fast violet stain and immunohistochemical method (using polyclonal rabbit anti-*Hp* antibody (dilution 1:50), DAKO). Therefore, we should keep our eyes "wide open" and not "wide shut" in the glaucoma-*Hp* association.

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Conflict of interest statement
None declared.

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Author reply: *Helicobacter pylori* and glaucoma: A mission impossible!

Dear Editor,

We really thank Prof. Kountouras and colleagues for their letter which give us the possibility to further comment on *Helicobacter pylori* and glaucoma association [1]. We would congratulate the Authors who were able to find further studies (from Italy, Iran, Turkey, and China) on such a field, which are unavailable on PubMed. However, in Table 1, they provided incomplete information regarding these studies. Indeed, the clinicians would know how many patients were enrolled, what test for *H. pylori* diagnosis was used, the statistical difference between groups, instead of simply knowing whether a 'positive' or 'negative' association is present. Only by evaluating all these data, it is possible to assess the reliability of each study. In other words, a 'positive' association based on a biased study is not equivalent to a 'negative' association based on a well performed study.

The Authors listed a series of alterations regarding cytokines, platelets, monocytes, and vascular function by which *H. pylori* would be involved in glaucoma pathogenesis. However, the proof of a medical association cannot be achieved by multiplying the hypotheses, but providing data. Indeed, although these hypotheses are fascinating, none has proven to have a significant role in glaucoma pathogenesis. In Medicine – as well as in other scientific fields – all assumptions need to be experimentally verified, otherwise we are performing a mere philosophic debate with the consequent risk of putting back 'the clock of scientific history' to before Galileo Galilei! In detail, the main factor involved in glaucoma pathogenesis is intra-ocular hypertension, and all medical and surgical approaches currently used are effective because of an impressive reduction of intra-ocular pressure. To date, there are no studies – which fulfil Koch's postulates – demonstrating that intra-ocular hypertension is caused by *H. pylori* infection.

Although we can agree that some of the cited potential alterations caused by *H. pylori* may have a role – if proven – in optical neuropathy, the hypothesized 'voyage' of *H. pylori* from the mouth to the eyes is unrealistic, and the mouth is not a definite reservoir for viable *H. pylori* bacteria. Generally, only fragments of bacterial DNA have been isolated from dental plaque, whilst *H. pylori* has been rarely cultured from saliva or the oral cavity [2]. This is due to obligate tropism of *H. pylori* for gastric epithelium which is lacking in the mouth [3]. Anyhow, the bacterium would need to climb the nasolacrimal duct to reach the conjunctiva, then penetrate into the aqueous humour. Is there sufficient evidence that viable *H. pylori* bacteria are present on the surface of the iris and trabeculum of glaucomatous eyes as the Authors supposed in a single study [4]? We already pointed out that the coccoid images (bacteria?) – which were present in only a minority (11.6%) of glaucoma patients – were not fully characterized as *H. pylori* with immunological assessment [1]. The lack of gastric mucosa inlets in the eyes, and the absence of active inflammation on the provided histological images further refute the presence of viable *H. pylori* bacteria. Therefore, the passage of *H. pylori* from mouth to the eyes seems to be a 'mission impossible'.

Conflict of interest statement
None declared.

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Helicobacter pylori, oxidative stress and glaucoma

Dear Editor

We read the editorial on primary open-angle glaucoma (POAG) and *Helicobacter pylori* by Zullo et al. with great interest [1]. The authors investigated existing data regarding a possible association between the two diseases and concluded that there is limited evidence supporting the role of *H. pylori* in glaucoma pathogenesis.

COMMENTS AND RESPONSES

Comment on: Jeon et al. *Helicobacter pylori* Infection Is Associated With an Increased Rate of Diabetes. Diabetes Care 2012;35:520–525

Jeon et al. (1) stated that infectious agents might have an impact on cardiovascular disease (CVD) and metabolic syndrome, potentially mediated by increased inflammatory markers, including C-reactive protein (CRP) and interleukin (IL)-6. However, epidemiological studies investigating the impact of pathogen burden on diabetes have been limited; cross-sectional studies examining systemic pathogens and insulin resistance (IR) or prevalent diabetes have produced equivocal findings, and *Helicobacter pylori* infection (*Hp*-I) shows no association with IR or prevalent diabetes.

We previously conducted a systematic review summarizing the epidemiological evidence regarding the association between *Hp*-I and IR quantitative-only indices (2). A positive association between *Hp*-I and homeostasis model assessment of insulin resistance (HOMA-IR), used to quantify IR in all nine selected studies, appears to exist. More specifically, when the study groups were divided according to *Hp* status (negative or positive), higher HOMA-IR was found in all but one study (2).

The authors (1) reported that individuals who developed diabetes had a higher HOMA-IR, but not IL-6 and CRP, than those who did not develop diabetes; however, the association between *Hp*-I and diabetes incidence remained significant after adjustment for HOMA-IR, IL-6, and CRP, indicating that *Hp*-I may affect diabetes independently of these factors. Nevertheless, glucose, insulin, CRP, and IL-6 were measured at baseline and not when each individual was diagnosed with diabetes. HOMA-IR, IL-6, and CRP might have been significantly higher when the diagnosis of diabetes was established than in the baseline assessment, and *Hp*-I might not have been associated with diabetes independently of these factors.

Hp-I may influence the pathophysiology of IR and IR syndrome, including diabetes, through several possible mechanisms (2,3): *1*) *Hp*-I releases large amounts of proinflammatory and vasoactive substances, such as cytokines (IL-1, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor [TNF]-α, interferon-γ), eicosanoids (leukotrienes, prostaglandins), and acute-phase proteins (CRP, fibrinogen). Fibrinogen appears to be significantly higher in diabetic patients with CVD, and our and others' series (2) demonstrate that increased fibrinogen levels are associated with *Hp*-I and can be significantly reduced by *Hp* eradication. *2*) *Hp*-I promotes platelet activation and aggregation and increases various proatherogenic factors including homocysteine, a risk factor for type 2 diabetes, obesity, and CVD. *3*) *Hp*-I produces reactive oxygen species and increases circulating concentrations of lipid peroxides, also associated with diabetes and CVD. *4*) Finally, *Hp*-I influences the apoptotic process. *Hp* could indirectly affect the pancreas and other target organs, e.g., the heart, through the release of numerous cytokines, such as TNF-α, acting at a distance (TNF-α–mediated β-cell dysfunction and apoptotic destruction in human islets) (4,5). Besides, *Hp* vacuolating cytotoxin stimulates apoptosis via a mitochondria-dependent pathway (5) and downregulation of antiapoptotic Bcl-2, upregulation of proapoptotic Bax, and increased activation of caspase-9 and -3 also indicate that apoptosis in patients with diabetes occurs via an intrinsic mitochondrial pathway.

However, further studies are needed to elucidate in depth the role of the aforementioned parameters in the pathophysiology of IR/diabetes-related disorders and the potential treatments of diabetes inferred from the *Hp*-related pathophysiology.

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ORIGINAL PAPER
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Oncogenes' Expression in Greek Patients with Primary Open-Angle Glaucoma in Association with *Helicobacter pylori* Status

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Abstract

Background & Aims: An association between *Helicobacter pylori* (*H. pylori*) infection and glaucoma has been previously suggested. The aim of this study was to investigate the apoptotic and cell proliferation processes as potential common pathogenetic mechanisms underlying this association.

Methods: Fifty-one glaucoma patients (33 female, mean age: 71.4±9.2 years) were initially enrolled. Biopsy specimens obtained on upper gastrointestinal (GI) endoscopy were evaluated for *H. pylori* presence, for expression of genes involved in apoptosis and cell proliferation (Ki-67, p53, Bcl-2), and for indices of cellular immune surveillance [T-lymphocytes (TLs) and B-lymphocytes (BLs)].

Results: Of the 43 patients eligible for upper GI endoscopy, 90.7% were tested positive for *H. pylori* infection. Ki-67 was positively expressed in 81.25% patients with *H. pylori* infection and in 1 patient without *H. pylori* infection. p53 was positively expressed in 31.25% patients with *H. pylori* infection but not in the patients without *H. pylori* infection. Bcl-2 was positively expressed in 68.75% patients with *H. pylori* infection and in 1 patient without *H. pylori* infection. Ki-67, p53 and Bcl-2 were over-expressed in 19%, 25% and 37.5%, respectively, of the patients with *H. pylori* infection, but none of them was over-expressed in the patient without *H. pylori* infection. TL marker was positively expressed in all patients with *H. pylori* infection and in the one patient without *H. pylori* infection. BL marker was positively expressed only in one patient with this infection.

Conclusion: *H. pylori*-induced oncogenes Ki-67, p53 and Bcl-2, and TLs involved in apoptotic and cell proliferation pathways might contribute to glaucomatous neuropathy, with an additional oncogenic potential.

Immunogastroenterology 2012; 1:40-46

Key words

Helicobacter pylori; primary open-angle glaucoma; oncogenes; apoptosis; immunohistochemistry

Introduction

By using histology in gastric biopsy specimens, our team has previously documented for the first time a high prevalence of *Helicobacter pylori* (*H. pylori*) infection in Greek patients with primary open-angle (POAG) and pseudo-exfoliation glaucoma.[1] In 3 subsequent studies, we reported: a) a beneficial effect of *H. pylori* eradication on glaucoma progression, suggesting a possible causal link between the bacterium and glaucoma;[2] b) increased *H. pylori*-specific IgG antibody concentration in the aqueous humor of POAG patients; the concentration of this antibody correlated with the degree of vertical cupping, possibly indicating the severity of glaucomatous damage;[3] and c) histological presence of *H. pylori* bacteria in incisional biopsy specimens obtained from the trabeculum and iris of patients with POAG during trabeculectomy surgery.[4] Reports from other ethnic populations also showed a relationship between glaucoma and *H. pylori* infection,[5,6] although this has not been confirmed by all the relevant studies published so far,[7,8] and thus this association may only apply to a limited sub-population of glaucoma patients; similar observations have been made in Korea, China, India, Turkey, and Iran.[6,9,10]

Furthermore, we have previously provided comprehensive reviews of a series of pathogenetic mechanisms potentially implicated in the *H. pylori* – glaucoma association. Summarizing these mechanisms, *H. pylori* infection may influence the pathophysiology of glaucoma by: promoting platelet and platelet-leukocyte aggregation, releasing proinflammatory and vasoactive substances,[11,12] and affecting the apoptotic process, which may be also involved in the pathogenesis of glaucomatous neuropathy.[13,14]

We herein further investigated our previous notion that apoptosis and cell proliferation might be common pathogenetic mechanisms underlying these two entities (*H. pylori* infection and glaucoma), by evaluating oncogenes, onco-suppressive genes associated with apoptosis, or anti-apoptotic proteins and cellular indices of immune surveillance in Greek patients with POAG and *H. pylori* infection.

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Table 1. Percentage of positively stained epithelial cell nuclei for p53, Ki-67 and Bcl-2

Percentage of stained epithelial cell nuclei	Stain grade
0-9%	0
10%-49%*	1
≥50%	2

*Positive samples were considered those with a stained epithelial cell nuclei percentage of ≥10%.

Patients and Methods

Fifty-one consecutive patients (33 female, mean age: 71.4±9.2 years, range 50-84 years) with POAG were initially enrolled in this study, according to the following inclusion criteria: a history of intraocular pressure of 21 mmHg or more; typical optic nerve head changes including saucerization, rim thinning or notching in the inferior or superior temporal area of the optic nerve head, or total glaucomatous cupping; and typical visual field loss including a paracentral, arcuate, or Seidel's scotoma or a nasal step. All POAG patients had prior experience with automated perimetry. The visual fields were assessed with the Octopus program G1 (Octopus 500EZ G1 program, Luterzeag AG, Zurich, Switzerland) by the same perimetrist (G.S.). We excluded patients with closed angle glaucoma, secondary glaucoma, previous eye surgery, previous argon-laser trabeculoplasty, and young patients less than 30 years of age. Patients with myopia or hypermetropia greater than 3 D or astigmatism more than 1 D that could potentially affect our measurements were also excluded. Only topical glaucoma treatment was allowed in the POAG patients included in this study. None of the patients received any oral drug that could decrease intraocular pressure (e.g., carbonic anhydrase inhibitors).

The presence of *H. pylori* was established through an upper gastrointestinal (GI) endoscopy and histology, however 8 patients were deemed not suitable endoscopy candidates or refused to undergo an endoscopy, and were subsequently excluded from this study. Thus, 43 POAG patients (26 female, mean age: 71.4±8.9 years, range 50-84 years) were finally included in this study.

Specifically, POAG patients who underwent an upper GI endoscopy, reported at 9 a.m. after a 12-hour fast. Intravenous sedation was given, and standard upper GI endoscopy was performed with a forward viewing endoscope Olympus GIF type V2 (Olympus Optical, Tokyo, Japan) for evaluation of any macroscopic abnormalities. Simultaneously, two biopsy specimens were obtained (Multibyte biopsy forceps, Boston Scientific, Microvasive, Watertown, MA, USA) from the antral region within 2 cm of the pyloric ring and two from the corpus, and they were placed in 10% formalin and submitted for histological examination. To prevent contamination of specimens taken from different sites, biopsy specimens from each site were taken with a fresh pair of sterile forceps. The forceps were wiped with alcohol on withdrawal from the endoscope to remove any organism that might have been present in the biopsy channel. Endoscopes were sterilized between procedures according to standard guidelines, mentioned previously.[1]

All specimens were stained with hematoxylin and eosin, and Cresyl fast violet and/or Giemsa (for detection of *H. pylori* organisms) stain. The same experienced pathologist (I.V.) assessed all the specimens.

Biopsy specimens obtained on upper GI endoscopy were evaluated by immunohistochemistry for the expression of: i. oncogene Ki-67; ii. oncosuppressive gene p53, involved in apoptosis; iii. anti-apoptotic gene Bcl-2; and iv. cellular indices of immune surveillance by measuring T-lymphocytes (TLs, Ubiquitin carboxy-terminal hydrolase L1, UCHL-1) and B-lymphocytes (BLs, CD20).

The antibodies employed were currently available for *in vitro* diagnostic use (Biogenex Laboratories, Inc., San Ramon, CA, USA). These antibodies were designed for the specific localization of Ki-67, p53 and Bcl-2 antigens in formalin-fixed, paraffin-embedded tissue sections. The demonstration of antigens by immunohistochemistry is a two-step process involving first, the binding of a primary antibody to the antigen of interest, and second, the detection of bound antibody by a chromogen. After following the manufacturer's instructions for preparation of the slides, they were loaded on the Optimax cell stainer (Optimax-plus, Biogenex), and the percentage of positively stained epithelial cell nuclei for p53, Ki-67 and Bcl-2 was measured. All the samples were semiquantitatively scored as: 0 (negative), 1 (weakly positive), 2 (positive). This scale was rearranged into a two point scale: 0 (negative) and 1 (positive). Positive samples were considered those with a stained epithelial cell nuclei percentage of ≥10% (**Table 1**).

For the evaluation of the expression of BLs and TLs, the following antibodies were employed: 1) anti-CD20 (BLs) (BioGenex, San Ramon, CA, USA). The CD20 antigen, also known as B1, is a 33-35 kD glycoprotein found on all BLs in peripheral blood, lymph node, spleen, tonsil, and bone marrow; and 2) UCHL-1 (BioGenex, San Ramon, CA, USA). This antibody recognizes a 185 kD molecule (CD45RO) which occurs on mature activated TLs, most thymocytes, and a sub-population of resting TLs within both the CD4 and CD8 subsets. UCHL-1 shows no reactivity with normal B or NK cells, but will react with granulocytes and monocytes.

The same experienced pathologist (I.V.), masked to the presence of *H. pylori*, assessed the expression of the oncogenes in all specimens.

The study was designed according to the principles of the Declaration of Helsinki (1964) and the study protocol was approved by the local ethics committee. All patients gave written informed consent prior to their enrolment in the study.

Results

The epidemiological data of the 43 POAG patients enrolled are given in Table 2. Mean duration from establishing glaucoma diagnosis was 6.9±6.6 years, and, despite continuous treatment with topical anti-glaucoma therapy, mean intraocular pressure was 25.8±9.2 mmHg in the right and 24.2±10.9 mmHg in the left

Table 2. Epidemiological data of patients with primary open-angle glaucoma (POAG)

Parameters	POAG patients (n=43)
Age (Mean±SD (range), years)	71.4±8.9 (49-93)
Sex (Male/Female)	17/26
Presence of *H. pylori* infection by histology	39/43 (90.7%)
Duration of glaucoma (years)	6.9±6.6
Mean IOP in right eye (mmHg)	25.8±9.2
Mean IOP in left eye (mmHg)	24.2±10.9
Mean visual acuity (left) (/10)	5.2±3.1
Mean visual acuity (right) (/10)	4.9±3.2

IOP, intraocular pressure.

Table 3. Percentage of positively stained epithelial cell nuclei expressing p53, Ki-67 and Bcl-2, and expression of B-lymphocytes (BLs, CD20) and T-lymphocytes (TLs, UCHL-1) in 17 primary open-angle glaucoma (POAG) patients

Oncogene	*H. pylori*-positive POAG patients (n=16)	*H. pylori*-negative POAG patient (n=1)
BLs (CD20)	1 (6.25%)	0
TLs (UCHL-1)	16 (100%)	1
Ki-67	13 (81.25%)	1
0-9%	3 (18.75%)	0
10%-49%	10 (62.50%)	1
≥50%	3 (18.75%)	0
p53	5 (31.25%)	0
0-9%	11 (68.75%)	1
10%-49%	1 (6.25%)	0
≥50%	4 (25%)	0
Bcl-2	11 (68.75%)	1
0-9%	5 (31.25%)	0
10%-49%	5 (31.25%)	1
≥50%	6 (37.5%)	0

UCHL-1, ubiquitin carboxy-terminal hydrolase L1.

eye, negatively affecting visual acuity (mean value about 5/10) in both eyes.

Forty-three POAG patients were eligible and agreed to undergo a selective upper GI endoscopy; five had advanced age and serious respiratory comorbidities and were deemed not suitable endoscopy candidates; and the remaining 3 refused to undergo an upper GI endoscopy. All 8 POAG patients in whom biopsies were not obtained were excluded from the study. Thirty-nine of 43 (90.7%) POAG patients were tested positive for *H. pylori* infection by histology (**Table 2**).

Oncogenes' expression in 17 of 43 POAG patients, in association with *H. pylori* presence, is demonstrated in **Table 3**. Specifically, oncogene Ki-67 was positively expressed (stain grade 1 or 2) in 13 of 16 (81.25%) POAG patients with *H. pylori* infection and in 1 POAG patient without *H. pylori* infection (grade 1). Onco-suppressive gene p53 was positively expressed in 5 of 16 (31.25%) POAG patients with *H. pylori* infection but not in the POAG patient without *H. pylori* infection (grade 0). Anti-apoptotic protein Bcl-2 was positively expressed in 11 of 16 (68.75%) POAG patients with *H. pylori* infection and in 1 POAG patient without *H. pylori* infection (grade 1). Finally, Ki-67, p53 and Bcl-2 were over-expressed (grade 2) in 19%, 25% and 37.5%, respectively, of the POAG patients with *H. pylori* infection, but none of them was found over-expressed in the POAG patient without *H. pylori* infection.

UCHL-1 was positively expressed in all POAG patients with *H. pylori* infection and in the one POAG patient without *H. pylori* infection. Concerning BLs, CD20 was positively expressed only in one POAG patient with *H. pylori* infection.

Discussion

Our findings show for the first time a positive expression of oncogenes (Ki-67), onco-suppressive genes associated with apoptosis (p53), and anti-apoptotic proteins (Bcl-2) in the gastric mucosa of *H. pylori*-positive POAG patients.

More specifically, over-expression of Ki-67 (percentage of stained epithelial cell nuclei ≥50%, stain grade 2) was observed in 1/4 of our patients with POAG and *H. pylori* infection. Ki-67 oncogene, over-expressed in proliferating cells, has been shown to upregulate cellular proliferation and oncogenesis. At the upper GI tract level, over-expression of Ki-67 in the esophageal epithelium was shown in advanced reflux esophagitis and in Barrett's esophagus.[15] The persistent Ki-67 expression in the mucosa of Barrett's esophagus with dysplasia might be a useful diagnostic index for the expression of the dysfunction of the regulatory mechanism of the cell cycle that leads to increased cell proliferation, neoplastic transformation, high-grade dysplasia and

finally development of invasive adenocarcinoma;[16] a gradually increasing concentration of Ki-67 has been shown in the sequence of Barrett's esophagus – low-grade dysplasia – high-grade dysplasia – esophageal adenocarcinoma from 25% up to 87%.[17] Likewise, an increase in Ki-67 has been demonstrated during the transition from gut metaplasia to gastric cancer, a sequence closely associated with *H. pylori* infection.[18] Ki-67, an index showing proliferation of the gastric epithelium and which is induced by *H. pylori* infection and CagA-positive strains, is significantly associated with the inflammatory host reaction.[19] At the eye level, Ki-67 has been used as an index of melanoma proliferation[20] and as an index showing response to therapy with agents that inhibit the proliferation of Tenon's fibroblasts, decreasing the excessive scarring after trabeculectomy.[21]

Regarding glaucoma pathogenesis, recent experimental data indicate that cell proliferation rather than astrocyte hypertrophy, characterizes early pressure-induced optic nerve head (ONH) injury; ONH is the principal site of initial axonal injury in glaucoma.[22] In view of these data, it is reasonable to speculate that *H. pylori*, upon reaching the eye, might induce Ki-67 expression and the aforementioned cell proliferation, thereby possibly initiating the glaucomatous process at least in the proportion of the patients with Ki-67 over-expression. However, further relative studies are needed to elucidate this speculation.

Over-expression of p53 was observed in 1/3 of our POAG patients with *H. pylori* infection. This nuclear onco-suppressive protein is implicated in maintaining the genome integrity: DNA damage leads to an increased p53 expression, which causes arrest of the active mitotic cycle in phase G1. Thereafter, p53 induces factors which allow DNA repair, while, in case of a severe DNA damage, it induces factors promoting apoptosis.[23] In this regard, since apoptosis also plays a crucial role in glaucoma development and progression, it is also reasonable to further speculate that when *H. pylori* reaches the eye, it might induce p53-related retinal ganglion cell apoptosis, at least in the proportion of the POAG patients with p53 over-expression; the arginine variant of rs1042522 within p53 has increased apoptotic potential and is associated with increased risk of POAG.[24] Again, further relative research is mandatory to confirm this hypothesis.

Besides, mutations of p53 are considered of critical importance and have been studied extensively, since they are found in the majority of the neoplasms.[23] For instance, the p53 pathway is inactivated in retinoblastoma with subsequent inhibition of apoptosis.[25]

Over-expression of the anti-apoptotic Bcl-2 was observed in 3/4 of our POAG patients with *H. pylori* infection. The role of the Bcl-2 family of proteins in retinal degeneration is still controversial. However, alteration in Bcl-2-related proteins has been observed in several models of retinal injury. In particular, the pro-apoptotic Bax protein has been suggested to play a critical role in apoptotic pathways in murine glaucoma model. Pro-apoptotic Bax α and β isoforms are upregulated in diseased retina. This is associated with a progressive reduction in the anti-apoptotic Bcl-2, reflecting imbalanced Bcl-2/Bax ratio as the disease progresses.[26] In this respect, relative research has suggested that mitochondria-dependent apoptosis signaling might contribute to apoptosis in a rat model of glaucoma involving chronic increases in intraocular pressure; mitochondria-dependent signaling involves increases in mitochondrial membrane permeability and the mitochondrial release of factors that signal for cell degradation. Opening of a multi-protein, mitochondrial megapore contributes to the increased permeability and the anti-apoptotic Bcl-2 and Bcl-x_L proteins bind at the megapore and facilitate megapore closure and reduce the mitochondrial membrane permeability. Phosphorylated protein kinase B (Akt) serves as an integrator for cellular survival signals and facilitates the megapore actions of Bcl-2 and Bcl-x_L, which could protect retinal ganglion cells against insults that induce apoptosis. Several anti-apoptotic agents are evaluated for use in glaucoma, including brimonidine and propargylamines, which oppose mitochondria-dependent apoptosis through pathways involving phosphorylated Akt.[27] Specifically, deprenyl, a monoamine oxidase inhibitor used in the treatment of Parkinson's disease, along with its primary metabolite desmethyldeprenyl (DES), reduces neuronal apoptosis by a mechanism that requires gene transcription and involves the maintenance of mitochondrial membrane potential; DES increases mitochondrial Bcl-2 and Bcl-x_L levels and decreases the pro-apoptotic Bax levels, thereby inhibiting the permeability transition pore form opening and preventing the apoptotic degradation.[28]

Although in our study we did not estimate Bax expression, and this is a limitation that excludes the estimation of the mentioned Bax/Bcl-2 ratio, other relative data indicate that the upregulation effects of *H. pylori* infection on the pro-apoptotic Bax, are stronger than its induction of Bcl-2; this effect may increase apoptosis in patients with chronic gastritis.[29] If this is the case after *H. pylori*'s possible entry in eye, then we can speculate a similar increased apoptotic effect contributing to glaucoma development. Again, further relative studies are needed to clarify this field.

Besides, Bcl-2 family member proteins are known to be over-expressed in most cancers, including intraocular tumors, thereby playing a possible role in tumorigenesis.[30,31]

Expression of activated TLs and BLs was respectively observed in all and in only one of our POAG patients with *H. pylori* infection. Relative evidence indicates that *H. pylori* infection induces infiltration of TLs lymphocytes and triggers gastric inflammation to augment TL-mediated apoptosis leading to mucosal pathology.[32] Moreover, based on the immune cellular response to *H. pylori*, BLs appear to have a minor role in this response,[33] a finding comparable to that observed in this study. On the other hand, apart from inflammation, TLs and particularly CD4+ cells seem to be involved in carcinogenesis, while CD8+ cells have a minor or no role in carcinogenesis.[33] If this is the case after *H. pylori*'s possible entry in eye, then we can also speculate a similar TL-mediated apoptotic effect contributing to glaucomatous neuropathy[34] with potential oncogenic effect.

Initially, we had proposed the following pathological mechanisms by which *H. pylori* might elicit glaucomatous neuropathy (**Figure 1**): 1) promoting platelet and platelet-leukocyte aggregation; 2) releasing pro-inflammatory and vasoactive substances, such as cytokines (interleukins 1, 6, 8, 10, 12, tumor necrosis factor (TNF)-α, interferon-γ), eicosanoids (leukotrienes, prostaglandins), and acute phase proteins (fibrinogen, C-reactive protein) involved in various vascular disorders (migraine, systemic hypertension, Raynaud's phenomenon, cardiovascular disease, and

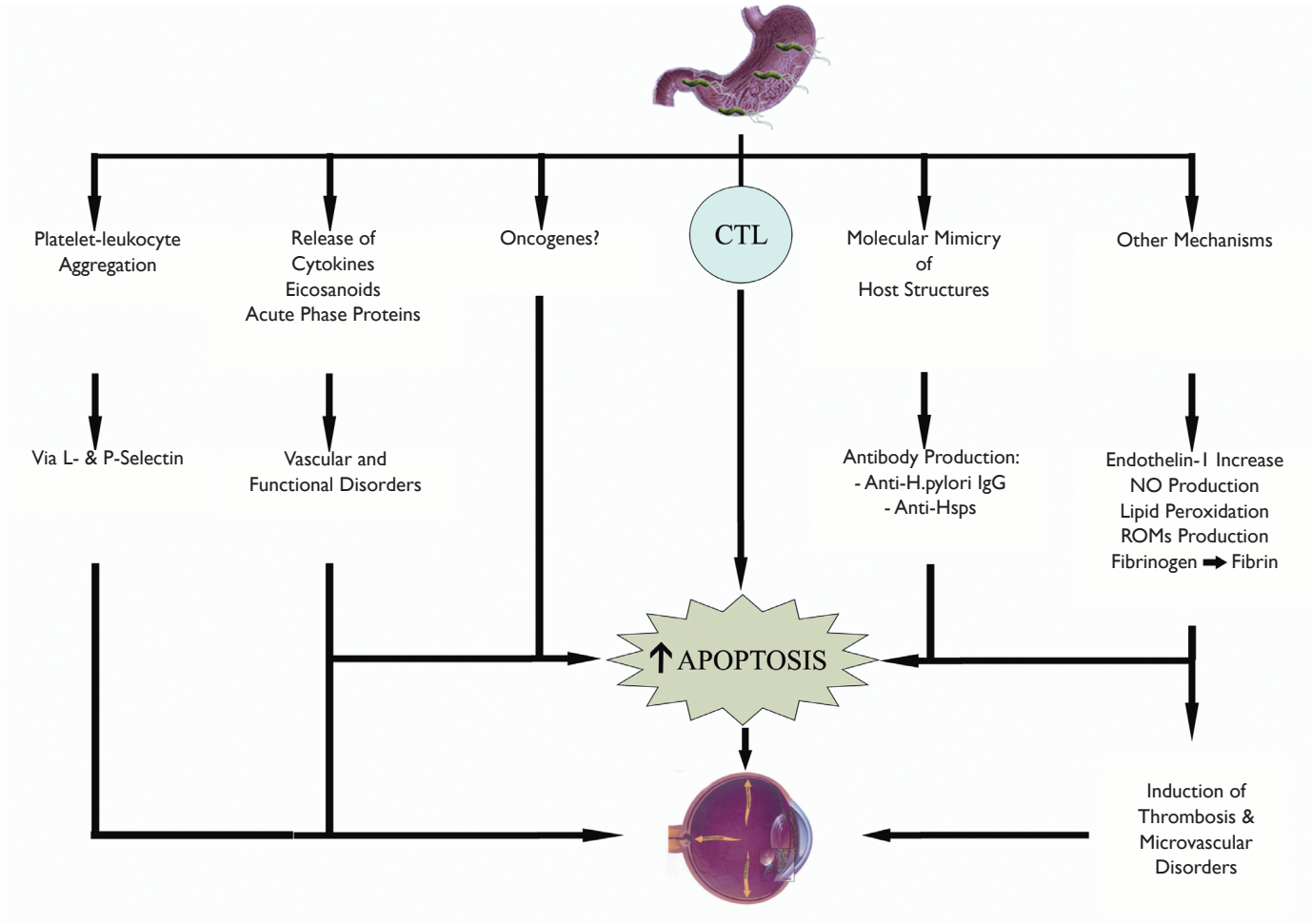

Figure 1. Summary of the proposed pathophysiological mechanisms by which *Helicobacter pylori* might elicit glaucomatous neuropathy, mainly through its involvement in the apoptotic process. CTL, cytotoxic T lymphocyte; anti-Hsps, heat shock protein antibodies; NO, nitric oxide; ROMs, reactive oxygen metabolites.

possibly glaucoma);[12] 3) stimulating mononuclear cells to induce a tissue factor-like procoagulant activity that converts fibrinogen into fibrin; 4) causing the development of cross mimicry between endothelial and *H. pylori* antigens; 5) producing oxidative stress and circulating lipid peroxides;[35] and 6) influencing the apoptotic process. These variables might also exert their own effects in the induction or progression of glaucomatous neuropathy[12] and other neurodegenerative disorders (Guillain-Barré syndrome, Parkinson's disease, Alzheimer's disease) associated with both *H. pylori* infection and glaucoma.[12,36-41]

Apart from the afore-mentioned mechanisms, *H. pylori* has been found in the nasal cavity[42] and mouth,[43] and the oral cavity might act as a permanent reservoir for this bacterium.[44] Recent studies showed that from the oral cavity, *H. pylori* may reach, through the nasal cavity, the anterior surface of the eye, causing blepharitis or worsening it, at least on the basis of cytological criteria.[45] Therefore, *H. pylori*'s intranasal route appears to explain, at least partly, the demonstrated presence of *H. pylori* in a few tissue samples obtained during trabeculectomy,[4] thereby having a major impact on understanding the role of this bacterium in the pathogenesis of POAG.

Moreover, specific antibodies are found in increased levels in glaucoma patients' sera, and when these antibodies access the brain due to blood brain barrier (BBB) disruption, they are capable of killing retinal cells, thereby contributing to glaucoma pathologies.[46] Specifically, comparable data could also be considered in the presence of blood-ocular barrier (BOB) dysfunction. In this respect, a series of factors have been implicated in inducing BBB disruption, including inflammatory mediators (e.g., cytokines and chemokines, induced by *H. pylori* infection) and oxidative stress.[46] *H. pylori* could indirectly affect the brain and other target organs, e.g. the heart or eye, through the release of numerous cytokines such as TNF-α acting at a distance; TNF-α is involved in BBB disruption through a mechanism involving matrix metalloproteinases' upregulation.[46] In addition, *H. pylori*-induced cytotoxin VacA exhibits chemotactic activities to the bone marrow-derived mast cells, and mast cells can be stimulated by corticotropin-releasing hormone, secreted under stress, to release mediators including histamine, interleukin 8, tryptase and vascular endothelial growth factor, which disrupt the BBB.[46] In this regard, as in the case of influx of activated monocytes infected with *Chlamydia pneumoniae* through the BBB involved in the pathogenesis of Alzheimer's disease, activated monocytes (possibly infected with *H. pylori* due to defective autophagy resulting in *H.*

pylori replication in autophagic vesicles)[47] or *H. pylori* antibodies circulating in the bloodstream might also enter the aqueous circulation due to BBB/BOB disruption, possibly contributing to glaucoma development and progression;[3] the *H. pylori* VacA promotes intracellular survival of the bacterium and modulates host immune responses.[48]

Providing evidence of *H. pylori*'s involvement in glaucomatous neuropathy, the results of the present series suggest that *H. pylori*-induced oncogenes Ki-67, p53 and Bcl-2, and TLs might contribute to glaucomatous neuropathy, with an additional oncogenic potential.

Conflicts of interest

The authors declared no conflicts of interest.

Summary Box

What is already known:

- A high prevalence of *Helicobacter pylori* (*H. pylori*) infection in Greek patients with primary open-angle (POAG) and pseudo-exfoliation glaucoma has been previously demonstrated.
- Recently histological presence of *H. pylori* bacteria was detected in biopsy specimens obtained from the trabeculum and iris of patients with POAG during trabeculectomy surgery.
- *H. pylori* infection may influence the pathophysiology of glaucoma by: promoting platelet and platelet–leukocyte aggregation; releasing proinflammatory and vasoactive substances; and affecting the apoptotic process.

What the new findings are:

- In a cohort of 51 POAG patients, the authors confirm their previous notion that apoptosis and cell proliferation might be common pathogenetic mechanisms underlying these two entities (*H. pylori* infection and glaucoma), by evaluating oncogenes, onco-suppressive genes associated with apoptosis, or anti-apoptotic proteins and indices of cellular immune surveillance in Greek patients with POAG and *H. pylori* infection.

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ORIGINAL PAPER
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Helicobacter pylori Immunomodulative Properties in a Mouse Model of Multiple Sclerosis

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Abstract

Background & Aims: *Helicobacter pylori* (*Hp*) has been epidemiologically linked to central nervous system diseases, including multiple sclerosis (MS), but experimental evidence on the field is lacking. The present study addresses the relative effect of *Hp*-strain SS1 (*Hp*-SS1) systemic immunization on the course of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS.

Methods: C57Bl/6 mice were immunized with *Hp*-SS1 antigen(s) in incomplete Freund's adjuvant (IFA) (n=8); with myelin oligodendrocyte glycoprotein peptide (MOG^{35-55}) and *Hp*-SS1 in IFA (n=8); with *Hp*-SS1 antigen(s) and complete Freund's adjuvant (CFA) (n=8); or with MOG^{35-55} and CFA (n=7). Three naive mice were also included. EAE clinical score and weight were daily evaluated. Proliferation of total spleen and draining lymph nodes' cells in the presence of *Hp*-SS1 antigen(s), concavalin A and MOG^{35-55}, was quantified.

Results: Mice immunized with *Hp*-SS1 did not show evidence of clinical disease. However, based on proliferation assay, mice immunized with *Hp*-SS1 antigen(s) in IFA exhibited similar proliferation when stimulated with MOG^{35-55}, to animals with classical EAE (1.21±0.021 vs. 1.18±0.059, p=0.425). Co-inoculation of *Hp*-SS1 antigen(s) with MOG^{35-55} in IFA, resulted in higher proliferation against MOG^{35-55}, compared to animals with classical EAE (1.7±0.344 vs. 1.18±0.059, p=0.450), although the difference did not reach statistical significance.

Conclusion: To our knowledge, this experimental approach for the first time indicates potential immunomodulatory properties of *Hp*-SS1 antigen(s) in mice with EAE. T-cell proliferation against MOG^{35-55} was evident in animals systemically inoculated with *Hp*-SS1 antigen(s), and co-inoculation of *Hp*-SS1 antigen(s) and MOG^{35-55} enhanced MOG^{35-55}-specific T-cell proliferation, compared to classical EAE.

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Key words

multiple sclerosis; experimental autoimmune encephalomyelitis; *Helicobacter pylori*; infection; environmental factor

Introduction

Autoimmune T-cell responses against antigens derived from the central nervous system (CNS) are thought to trigger several diseases, including multiple sclerosis (MS) which appears to occur in genetically predisposed individuals following exposure to environmental triggers, and account for 75% of the risk of developing MS.[1,2] Existing evidence from epidemiological studies highlight numerous infections as environmental MS triggers; infection can prime autoimmune T cells specific for CNS antigens, if the pathogen has molecular mimicry with CNS proteins.[3] Several pathogens have been under investigation, including Epstein-Barr virus,[4] *Chlamydia pneumoniae*[5] and Human Herpes Virus-6.[6]

Helicobacter pylori (*Hp*), a spiral gram-negative bacterium that colonizes human gastric mucosa,[7] has recently also been linked to a number of central and peripheral nervous system disorders including Alzheimer's disease (AD),[8] mild cognitive impairment,[9] glaucoma, defined as ocular AD,[10] Guillain-Barré syndrome (GBS),[11,12] and MS.[13,14] *Hp* has long been described to induce gastrointestinal disease by means of molecular mimicry,[15] a mechanism that may also be implicated in nerve damage;[16,17] *Hp* infection induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves, thereby contributing and possibly perpetuating neural tissue damage.[8,11-13] Moreover, eradicating *Hp* infection might alter the pathophysiology of neurodegenerative disorders possibly including MS.[8,13] However, the concept of a role of *Hp* infection in MS is based on a rather limited number of clinical studies yet lacking the support from experimental studies investigating the relative effect of *Hp* systemic immunization.

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Therefore, we aimed to test the hypothesis whether systemic immunization with *Hp*-SS1 antigen(s) modifies immunological reaction in mice with experimental autoimmune encephalomyelitis (EAE).

Materials and Methods

Bacteria and growth conditions

Hp-strain SS1 (*Hp*-SS1) was provided by the Hellenic Pasteur Institute. The mouse-adapted *Hp*-SS1 was derived from a clinical isolate associated with upper abdominal pain and peptic ulcer disease, as described by Lee et al.[18] *Hp*-SS1 has a prominent colonization capacity and an increased antigenic potential compared to other strains, owing predominantly to virulence factors, namely, vacuolating cytotoxin (VacA) and immunodominant cytotoxin-associated antigen (CagA),[19,20] as well as surface lipopolysaccharide (LPS) molecules that prime gastric acid stimulation.[21,22] *Hp*-SS1 was routinely subcultured on a blood agar (BA) medium supplemented with 10% horse blood, containing a *Hp*-SS1-selective antibiotic mixture,[18] and incubated under microaerobic conditions at 37 °C. Bacterial suspensions prepared from low-passage cultures of *Hp*-SS1 (i.e., cultures that have undergone 10 passages *in vitro*) were stored at 280 °C, in a tryptone casein soya broth solution containing 25% glycerol. For *in vivo* experiments, whole heat-inactivated bacteria in PBS were systemically inoculated, while for T-cell proliferation assay, *Hp*-SS1 antigen(s) was produced by sonication and subsequent dialysis (SpectraPor; cutoff pore size, 8 kDa).

Animals and experimental setting

Thirty-four C57Bl/6 mice were assigned in 5 experimental groups: group A, mice immunized with *Hp*-SS1 antigen(s) in incomplete Freund's adjuvant (IFA) (n=8); group B, mice inoculated with myelin oligodendrocyte glycoprotein peptide (MOG^{35-55}) and *Hp*-SS1 in IFA (n=8); group C, mice immunized with *Hp*-SS1 antigen(s) and complete Freund's adjuvant (CFA) (n=8); group D mice inoculated with MOG^{35-55} and CFA, according to the classical EAE protocol (n=7) and group E naive mice (n=3). Animal facilities and experimentation were subjected to The Animals Act 160/3.05.1991 applicable in Greece, and revised according to the 86/609/EEC/24.11.1986 directive, as well as Authorization for animal experimentation by local authorities.

EAE induction

EAE was conducted according to well-described MOG^{35-55} model in C57Bl/6 mice. Animals were purchased by the Pasteur Hellenic Research Institute and kept under specific pathogen free Conditions at the animal Facility of the Laboratory of Neuroimmunology of the Second Department of Neurology in AHEPA General Hospital in Thessaloniki. Animals were fed a normal diet and were given free water. Animal experimentation was conducted on the basis of the approval of our Institute's Ethics Committee and in accordance with the Greek Public Health Service Policy on Human Care and Use of Laboratory Animals. EAE was induced in 6-7 week old female C57B/6 mice by immunization with 100 μL of PBS emulsion containing 0.15 μg of purified MOG^{35-55} (MEVGWYRSPFSRVVHLYRNGK, one-letter sequence code), 0.4 mg whole inactivated *Hp*-SS1 bacteria or both, according to the experimental group, and an equal volume of IFA or CFA, containing 0.4 mg *Mycobacterium tuberculosis* (H37RA), according to the experimental group. Therefore, 0.2 mL total volume was injected subcutaneously at the base of the tail, at day of induction (day 0) and at day 7. In addition, 300 ng of Bordetella pertussis toxin in 0.2 mL PBS was injected intraperitoneally at day of induction and at day 2. Animals were daily evaluated in terms of clinical score and weight loss or gain. Animals were followed up to 25 days post-induction (dpi).

Clinical assessment of EAE was made according to an established scale from 0 to 6 as follows: 0: asymptomatic; 1: partial loss of tail tonicity; 2: atonic tail; 3: hind leg weakness and/or difficulty to roll over; 4: hind leg paralysis; 5: four leg paralysis; and 6: death due to EAE.

In vitro *proliferation assay*

Spleen and draining lymph nodes were excised from two mice per immunization experimental group and one naive mouse, 10 days post-immunization. Harvested organs were pooled per group and cultured as single-cell suspensions. The proliferation of derived T-cells was assayed *in vitro* by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) incorporation in response to 1 μg/mL concanavalin A (ConA), 10 μg/mL MOG^{35-55} or 10 μg/mL *Hp*-SS1 antigen(s), in five wells per condition. *Hp*-SS1 antigen(s), produced by sonication and subsequent dialysis (SpectraPor; cutoff pore size, 8 kDa), was provided by the Hellenic Pasteur Institute. Optical density was measured by the use of spectrophotometer at 450 nm. Relative T-cell proliferation in the presence of conA, MOG^{35-55} or *Hp*-SS1 antigen(s) was estimated with reference to plain (unstimulated) T-cells proliferation of the respective experimental group, and was expressed as stimulation index (SI) [SI=OD value (sample)/ OD value (unstimulated cells)].

Statistical analysis

Student's t-test and One-Way ANOVA was used for the comparison of mean EAE scores, mouse weight and *in vitro* total splenocytes' proliferation between experimental groups. Statistical significance was set at the level of $p \leq 0.05$. Values represent mean±standard deviation (SD). Analysis was conducted by the use of SPSS for Windows (18.0, Chicago IL, USA).

Results

Systemic immunization with *Hp*-SS1 antigen(s) in the absence of MOG^{35-55} is not encephalitogenic though immunogenic. Group D animals exhibited severe EAE with high mean maximal score (4.5±0.57), day of disease onset 14.8±1.6 and mean Area Under the Curve 42.0±8.9. Groups A, B and C did not develop clinical signs of EAE (**Fig. 1A**). One mouse in group A (1/8) and one in group B (1/8) died on day 9 post-induction. In addition, animals of group B were sweat and exhibited piloerection, similarly to group D. Animals of group D exhibited a typical weight gain and loss for EAE (on days 10 and 19 respectively), while groups A, B and C gained weight on day 10 which was

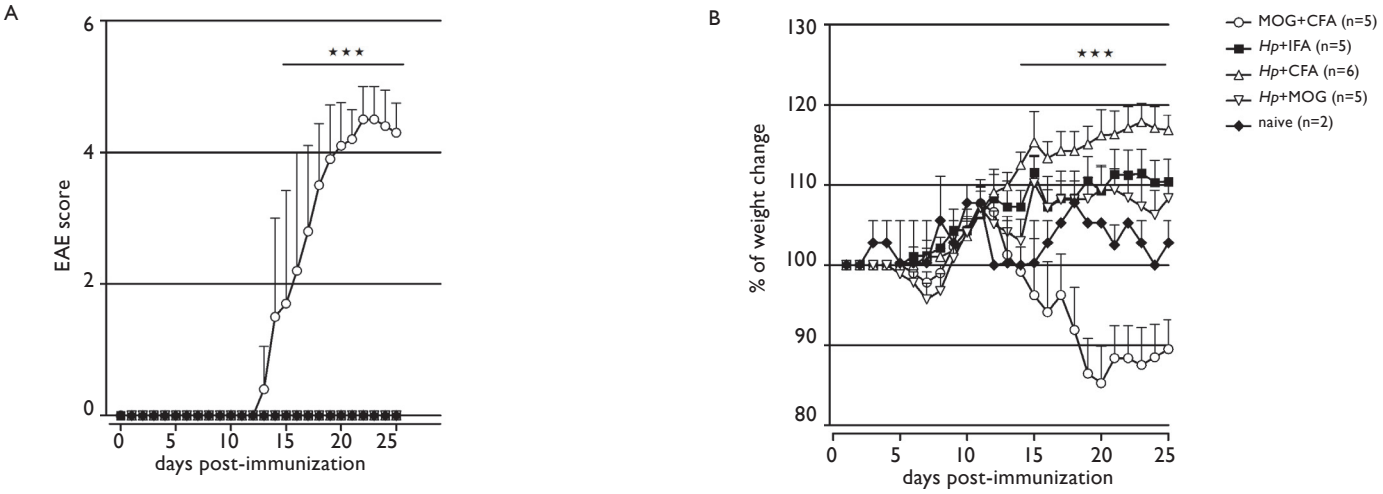

Figure 1. Mean EAE score (A) and % change of weight relative to day 0 (B) of mice inoculated with MOG$^{35-55}$ and CFA (MOG+CFA), according to the classical EAE protocol, mice immunized with *Hp*-SS1 antigen(s) in IFA (*Hp*+IFA), mice immunized with *Hp*-SS1 antigen(s) and CFA (*Hp*+CFA), mice inoculated with MOG$^{35-55}$ and *Hp*-SS1 in IFA (MOG+*Hp*) and naive mice. EAE, experimental autoimmune encephalomyelitis; MOG$^{35-55}$, myelin oligodendrocyte glycoprotein peptide; CFA, complete Freund's adjuvant; *Hp*-SS1, *Helicobacter pylori* strain SS1; IFA, incomplete Freund's adjuvant. ***represents p<0.001.

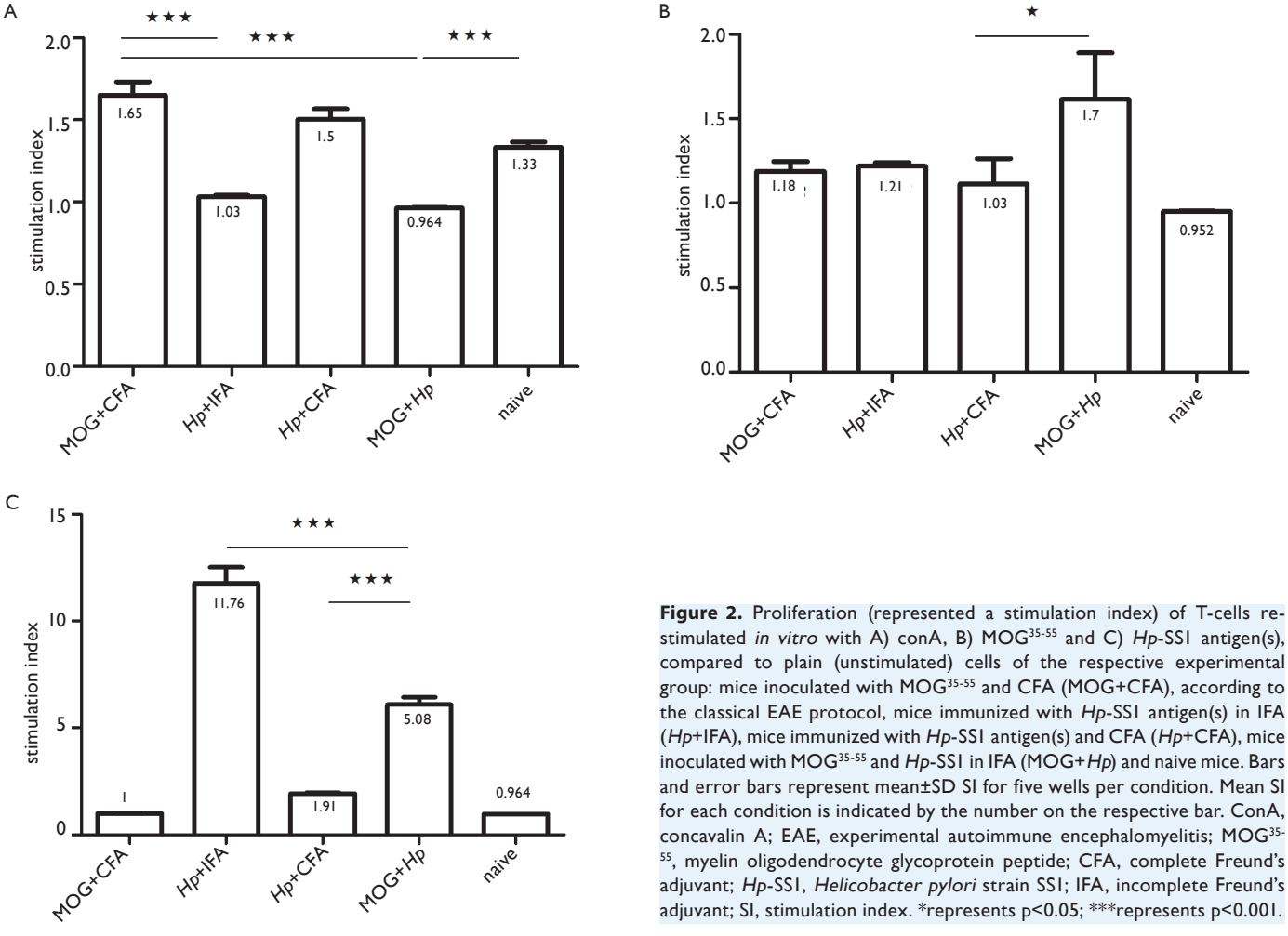

Figure 2. Proliferation (represented a stimulation index) of T-cells re-stimulated *in vitro* with A) conA, B) MOG$^{35-55}$ and C) *Hp*-SS1 antigen(s), compared to plain (unstimulated) cells of the respective experimental group: mice inoculated with MOG$^{35-55}$ and CFA (MOG+CFA), according to the classical EAE protocol, mice immunized with *Hp*-SS1 antigen(s) in IFA (*Hp*+IFA), mice immunized with *Hp*-SS1 antigen(s) and CFA (*Hp*+CFA), mice inoculated with MOG$^{35-55}$ and *Hp*-SS1 in IFA (MOG+*Hp*) and naive mice. Bars and error bars represent mean±SD SI for five wells per condition. Mean SI for each condition is indicated by the number on the respective bar. ConA, concavalin A; EAE, experimental autoimmune encephalomyelitis; MOG$^{35-55}$, myelin oligodendrocyte glycoprotein peptide; CFA, complete Freund's adjuvant; *Hp*-SS1, *Helicobacter pylori* strain SS1; IFA, incomplete Freund's adjuvant; SI, stimulation index. *represents p<0.05; ***represents p<0.001.

retained up to 25 dpi (**Fig. 1B**). It could be argued that *Hp*-SS1 antigen(s) administration accounts for the two mice that died on day 9 post-immunization, one of each *Hp*-SS1 inoculated experimental group. This is unlikely because the mice from the same groups that were followed over time did not show evidence of acute systemic abnormalities. Also, the mice that died did not

exhibit body weight loss until one day before. However, further experiments should verify this assumption.

To assess possible triggering effect of systemic immunization against *Hp*-SS1 antigen(s) in *ex vivo* cellular immune reaction, a T-cell proliferation assay at 10 dpi was conducted. Stimulation index of T-cells stimulated either with conA, MOG^{35-55}, or *Hp*-SS1 antigen(s), relative to unstimulated cells, as well as comparison to classical EAE and naive animals, is shown in **figure 2**. Animals immunized with *Hp*-SS1 in the absence of CFA, namely, groups A and B, exhibited reduced stimulation index, when T cells were triggered with conA, compared to naive animals (1.03±0.007 vs. 1.33±0.03, p<0.001; and 0.964±0.004 vs. 1.33±0.03, p<0.001, respectively). Similar results were present when the same groups were compared to classical EAE group (1.03±0.007 vs. 1.65±0.08, p<0.001; and 0.964±0.004 vs. 1.65±0.08, p<0.001, respectively). In animals immunized with *Hp*-SS1 antigen(s) in the presence of CFA, T-cell proliferation against conA was increased compared to naive animals (1.5±0.06 vs. 1.33±0.03, p<0.001). However, in the same group, T-cell proliferation against conA was reduced in comparison to classical EAE animals (1.5±0.06 vs. 1.65±0.08, p=0.015). As expected, a prominent T-cell proliferation against *Hp*-SS1 antigen(s) was present, in animals immunized with *Hp*-SS1 antigen(s) in IFA and *Hp*-SS1 antigen(s) and MOG^{35-55} in IFA (**Fig. 2**). Animals immunized with *Hp*-SS1 antigen(s) in CFA also exhibited a prominent, yet significantly reduced, T-cell proliferation against *Hp*-SS1 antigen(s), compared to animals that were immunized with either *Hp*-SS1, or *Hp*-SS1 and MOG^{35-55}, in IFA (1.91±0.049 vs. 11.76±.75, p<0.001; and 1.91±0.049 vs. 5.08±0.035, p<0.001 respectively).

Interestingly, mice immunized with *Hp*-SS1 in IFA exhibited similar T-cell proliferation against MOG^{35-55}, compared to classical EAE mice (1.21±0.021 vs. 1.18±0.059, p=0.425). T-cell proliferation against MOG^{35-55} in naive animals was reduced, compared to animals sensitized against *Hp*-SS1 in IFA (1.21±0.021 vs. 0.952±0.003, p=0.335), but the difference did not reach statistical significance. However, when *Hp*-SS1 was inoculated with CFA, T-cell proliferation against MOG^{35-55} was significantly reduced, compared to animals with classical EAE (1.03±0.15 vs. 1.7±0.344, p=0.013).

In mice immunized with *Hp*-SS1 and MOG^{35-55} in IFA, T-cell proliferation against MOG^{35-55} was increased compared to animals with classical EAE (1.7±0.344 vs. 1.18±0.059, p=0.450), although the difference did not reach statistical significance.

Discussion

To our knowledge, this is the first experimental attempt to study the possible immunomodulative properties of *Hp*-SS1 antigen(s) in EAE, the most widely used animal model of MS. So far, epidemiological evidence has linked *Hp* infection to MS, with molecular mimicry being the most prominent mechanism proposed.[13,19,20] However, experimental evidence in the field is lacking. In the present study, *Hp*-SS1,[18] an immunogenic strain common in humans, was subcutaneously inoculated to C57Bl/6 mice with EAE.

In our preliminary experimental setting, systemic immunization with *Hp*-SS1 antigen(s) did not prove to be encephalitogenic, administered either in IFA, or in combination with MOG^{35-55} or CFA. However, all animals were euthanized at the acute phase of the disease and were not followed to chronicity. The possibility that some animals would show clinical signs of disease after a longer follow-up cannot be ruled out. Therefore, future relative studies are needed to elucidate this field.

With respect to T-cell proliferation assay, we hereby provide evidence of immunomodulative properties of *Hp*-SS1 antigen(s), following systemic immunization of mice with EAE. *Hp*-SS1 suppressed T-cell proliferation as a response to panmitogenic reagent conA. This finding is in accordance with early reports of impaired cellular immunity in presence of surface bacterial LPS, as well as viral proteins.[23-25] However, the observed suppressive effect of *Hp*-SS1 immunization on T-cell proliferation, in response to conA, was less prominent when *Hp*-SS1 was inoculated with CFA.

On the other hand, we observed increased T-cell proliferation against MOG^{35-55} in animals immunized with *Hp*-SS1 and IFA, in the absence of systemic MOG^{35-55} administration. The proliferation rate observed here is similar to that observed in animals with classical EAE. The effect was counterbalanced in animals immunized with *Hp*-SS1 antigen(s) in the presence of CFA, where T-cell proliferation rate against MOG^{35-55} was significantly reduced compared to classical EAE. Furthermore, co-inoculation of *Hp*-SS1 antigen(s) and MOG^{35-55} in IFA, resulted in higher rate of T-cell proliferation against MOG^{35-55}, compared to animals with classical EAE.

Importantly, since T-cell proliferation against MOG^{35-55} was evident in animals not systemically inoculated with MOG^{35-55}, and co-inoculation of *Hp*-SS1 antigen(s) and MOG^{35-55} enhanced MOG-specific T-cell proliferation, compared to classical EAE, our findings may indicate presence of a T-cell population that cross-recognize *Hp*-SS1 and MOG epitopes; this cross-reaction might be relevant to MS pathophysiology. Moreover, the possibility of an *Hp*-SS1-MOG cross-recognizing clone is in accordance with existing knowledge of degenerate recognition by the T-cell receptor (TCR) in the process of formation of TCR-antigen-MHC trimolecular complex.[26] In addition, it has recently been reported that *Hp* infection seems to be one of the risk factors for the development of MS in a subset of patients, namely those being positive for serum anti-aquaporin-4 (anti-AQP4).[19] Bacteria harbor their own water channel proteins with some sequence homology to human AQP4. *Hp* exhibits water channels that serve as ion transporters. Namely, a putative water-filled channel important for transmembrane ion transfer has been described in the fourth transmembrane domain (TM4) of *Hp*.[27] Genes encoding functionally similar molecules are often highly conserved among different bacteria. To the extent that *Hp* water channels share sequence homology with human and bacterial AQP, molecular mimicry might be implicated to the *Hp*-associated pathology. Clinical evidence also supports this hypothesis. Up-regulated expression of AQP5 has been described in the cells of the intestinal type of adenocarcinoma,[28] a type of human gastric cancer that has been linked with *Hp* infection.[29] Therefore, molecular mimicry between human AQP and bacterial water channel molecules is worth exploring for

a potential source of cross-reactive antigens for the anti-AQP4 antibody. In this respect, *Hp*-neutrophil activated protein might be associated with the pathology of anti-AQP4 antibody-related neural damage in MS and neuromyelitis optica patients.[30] Moreover, molecular mimicry of host structures by the saccharide portion of LPS of the gastrointestinal pathogens *Campylobacter jejuni* (*C. jejuni*) and *Hp* is considered to be associated with the development of autoimmune sequelae in inflammatory neuropathies including GBS possibly associated with MS; chemical analyses of the core oligosaccharides of neuropathy-associated *C. jejuni* strains have revealed structural homology with human gangliosides.[31] Therefore, the aforementioned data indicate that *Hp* infection may be involved in the pathogenesis of MS, and molecular mimicry serves as a possible underlying mechanism.

It is reasonable to argue that, in order to support the hypothesis of molecular mimicry between *Hp*-SS1 antigen(s) and MOG, sequence homology should be studied. MOG^{35-55} was used for the induction of EAE as the highly encephalitogenic epitope for C57Bl/6 mice. If *Hp* shows molecular mimicry towards myelin antigens this does not necessarily apply to the specific MOG^{35-55} linear epitope. Instead, *Hp*-antigen-specific T cells may cross-react to conformational myelin epitopes unmasked by epitope spreading. It was recently shown that microbial peptides with limited sequence homology to MBP can induce MS-like disease in mice humanized for a TCR derived from an MS patient.[2] Therefore, although in silico studies are often used to demonstrate sequence homology between candidate cross-reacting epitopes, their results do not address the structural equivalence that is necessary for recognition by the TCR. However, the present study does not strictly address the hypothesis of molecular mimicry. The possibility that *Hp*-SS1 exerts its immunomodulative effect on EAE by providing an unspecific stimulus to the innate immunity should also be addressed.

In our results, proliferation to the plain cells in the presence of MOG^{35-55}, shows a lower range compared to the proliferation in the presence of conA or *Hp*-SS1 antigen(s). This proliferation range is difficult to be compared among the three conditions that were used for *in vitro* stimulation, namely, conA, MOG^{35-55} and *Hp*-SS1 antigen(s), due to the different pathway that these molecules follow to stimulate the T-cells. In this respect, discrimination between the proliferating T-cells and other cell types that are present when total splenocytes are plated, such as B-cells and other antigen-presenting cells, would be useful. ConA is a mitogenic factor, thereby inducing an increase in the proliferation rate of all cells. Similarly, *Hp*-SS1 antigen(s) contain LPS, which provide strong stimulus to the antigen-presenting cells. On the contrary, MOG^{35-55} reacts with antigen-specific TCR in a polyclonal T-cell population and the peptide affinity to the TCR can vary. Frequent low affinity TCRs have been described in polyclonal T cell populations, however these low affinity T-cells bear effector phenotype and contribute significantly to the cytokine pool produced.[32]

In conclusion, the present experimental setting indicates potential immunomodulatory properties of *Hp*-SS1 antigen(s) in mice suffering from EAE. Of note, proliferation of T cells per se does not necessarily imply their encephalitogenic or immunosuppressive properties. For this reason, characterization of the T-cell phenotype with respect to the cytokines produced would help to assign a specific T-cell lineage commitment. Further experiments are needed in order to study the effector properties of these cells. Future studies are needed to elucidate whether these properties are also evident when an *Hp*-SS1 infection model, rather than systemic immunization, is applied. Long-term assessment of *Hp*-SS1 immunomodulative effect in EAE is also mandatory, including cytokine evaluation, comparable effect of *Hp*-SS1 and other infectious factors (*Escherichia coli*), or possible synergistic effect of more than one *Hp*-SS1 strains in EAE.

Summary Box

What is already known:

- Epidemiological evidence links various viral and bacterial infections with multiple sclerosis (MS). Candidate pathogens currently under investigation are Epstein-Barr virus, *Chlamydia pneumoniae* and Human Herpes Virus-6.
- *Helicobacter pylori* (*Hp*) has recently been linked to a number of central and peripheral nervous system disorders including Alzheimer's disease (AD), mild cognitive impairment, glaucoma, defined as ocular AD, Guillain-Barré syndrome, and MS.
- *Hp* infection seems to be one of the risk factors for the development of MS in a subset of patients, namely those being positive for serum anti-aquaporin-4, and molecular mimicry of host structures by the saccharide portion of lipopolysaccharide of the gastrointestinal pathogens *Campylobacter jejuni* and *Hp* is considered to be associated with the development of autoimmune sequelae in inflammatory neuropathies. *Hp* infection may be involved in the pathogenesis of MS, and molecular mimicry serves as a possible underlying mechanism.

What the new findings are:

- Systemic immunization with *Hp*-strain SS1 (*Hp*-SS1) antigen(s) did not prove to be encephalitogenic, administered either in incomplete Freund's adjuvant, or in combination with myelin oligodendrocyte glycoprotein peptide MOG^{35-55} or complete Freund's adjuvant.
- With respect to T cell proliferation assay, we hereby provide evidence of immunomodulative properties of *Hp*-SS1 antigen(s), following systemic immunization of mice with experimental autoimmune encephalomyelitis.
- Our data indicate that *Hp* infection may be involved in the pathogenesis of MS, and molecular mimicry serves as a possible underlying mechanism. However, the possibility that *Hp*-SS1 systemic immunization exerts its immunomodulative properties via unspecific, non-antigen-dependent, activation of the innate immune reaction, cannot be excluded.

Contributors

Marina Boziki was responsible for experimental design, conducting in vivo *experiments, interpretation of data and preparing the manuscript. Nikolaos Grigoriadis was responsible for experimental design, critical revision and final approval of the manuscript. Georgia Deretzi was responsible for critical revision of the manuscript. Roza Lagoudaki conducted* in vitro *experiments and assisted in interpretation of data. Athanasios Lourbopoulos assisted in* in vivo *experiments and interpretation of data. Effrosini Panayotopoulou, Dionyssios Sgouras and Andreas Mentis provided* Hp-*SS1 material and were responsible for critical revision of the manuscript. Nikolaos Tascos was responsible for critical revision of the manuscript. Jannis Kountouras was responsible for conception, critical revision and final approval of the manuscript.*

Conflicts of interest

The authors declared no conflicts of interest.

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Letters to the Editor

RE: HELICOBACTER PYLORI *INFECTION AND COLORECTAL CANCER RISK: EVIDENCE FROM A LARGE POPULATION-BASED CASE-CONTROL STUDY IN GERMANY*

Zhang et al. (1) concluded that there is a serologic association between *Helicobacter pylori* (*H. pylori*) infection and the risk of colorectal cancer, especially for left-sided and early stage cancers, a finding that warrants confirmation and exploration of the underlying biologic mechanisms. However, as mentioned by the authors (1), the serologic measurement of infection status is less than perfect, which represents a specific limitation of their study.

Indeed, the serologic test does not discriminate between current and past infections and, apart from past infection that may even be more relevant for oncogenesis, such a distinction is essential because only current *H. pylori* infection induces humoral and cellular immune responses that induce or perpetuate chronic inflammatory processes in the gastrointestinal tract with potential oncogenic sequelae. Many cancers, including colorectal cancers, arise at the sites of chronic inflammation and infection (2, 3).

On the basis of histology, the practical gold standard for diagnosis of *H. pylori* infection, our own preliminary studies indicated *H. pylori* presence in malignant tissue in 34 of 41 (82.9%) patients with colorectal cancer (23 men; mean age, 73.6 years, standard deviation = 7.9 years) (4). It is important to note that in addition to using cresyl violet staining to detect *H. pylori*, we also documented its presence using an immunohistochemical method (using polyclonal rabbit anti-*H. pylori* antibody (dilution 1:50), DAKO, Athens, Greece) in malignant colonic tissues. Like Zhang et al. (1), we found a high incidence of left-sided cancers (29 of 41 patients (70.7%)). In addition, we found that the presence of *H. pylori* in malignant colonic tissue was associated with increased expression of the *Ki-67* oncogene in all tumor specimens and low expression in all adjacent tissue specimens (5). Moreover, p53 increased, and low expression was observed in 72.5% and 100% of tumor specimens and adjacent tissue specimens, respectively. Likewise, antiapoptotic Bcl-2 protein was observed in 60% and 9% of tumor specimens and adjacent tissue specimens, respectively, whereas proapoptotic Bax protein was observed in 9% and 100% of tumor specimens and adjacent tissue specimens, respectively (5). Therefore, *H. pylori* colonizing colonic tumor tissue seems to be associated with increased cell proliferation and impaired apoptotic process in malignant tissue compared with normal adjacent colonic mucosa, thereby contributing to colon cancer progression (5). In this regard, *H. pylori*-induced gastrin, which was also mentioned by the authors (1) as an oncogenic growth factor, shows antiapoptotic activity through upregulation of *Bcl-2* and contributes to gastric and colon carcinogenesis through stimulation of mutagenic and tumorigenic cyclooxygenase-2 expression (6).

Experimental data indicate that *H. pylori* infection leads to the development of chronic inflammation, hyperplasia, metaplasia, dysplasia, and recruitment and accumulation of bone marrow-derived cells (BMDCs), which may contribute to tumor formation in animal models with *H. pylori*-induced chronic gastric inflammatory process (2, 6). Because *H. pylori* also induces inflammatory changes in colonic mucosa, it would be reasonable to speculate that chronic *H. pylori* infection in humans also induces repopulation of the colon with BMDCs that might facilitate colon cancer development and progression (2, 6). In this regard, our own preliminary studies indicated increased expression of CD44 (a marker of human hematopoietic stem and progenitor cells) in malignant colonic tissue in 31 of our 41 patients (75.6%) with colorectal cancer (4). We also obtained comparable data for gastric cancer (2, 6). Therefore, these findings suggest the possible BMDCs involvement in *H. pylori*-associated colon and gastric cancer development and/or progression (2, 6).

ACKNOWLEDGMENTS

Conflict of interest: none declared.

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Editor's note: *In accordance with* Journal *policy, Zhang et al. were asked if they wished to respond to this letter, but they chose not to do so.*

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RE: "DERIVATION AND VALIDATION OF THE DENVER HUMAN IMMUNODEFICIENCY VIRUS (HIV) RISK SCORE FOR TARGETED HIV SCREENING"

Haukoos et al. developed a commendable risk score for prescreening for human immunodeficiency virus (HIV) testing in clinical settings (1). Their thoughtfully constructed model contains a number of important predictors of HIV infection. Although the authors concluded the contrary, the predictive utility of the model, as displayed in the receiver operating characteristics curve, seemed to us to strongly endorse the United States Centers for Disease Control and Prevention (CDC) guidelines for general HIV screening with highly sensitive tests (2).

The Denver sexually transmitted infection clinic data reflect a 0.5% prevalence of undiagnosed infection and an annual caseload of 10,000 patients (50 HIV-infected patients). Although values of sensitivity/specificity used to create Figure 2B in the article by Haukoos et al. were not reported, the joint maximum for both appears to be 80%/80% sensitivity/specificity on the curve for the derivation data. Applying the risk score at this cutpoint would result in referral of 2,030 patients for HIV-testing and detection of 40 HIV-positive patients, with 10 HIV-positive patients who received low risk scores not being tested. A value of the receiver operating characteristics curve that attains 95% sensitivity appears to occur at 20% specificity. Applying these properties, all but 2 HIV-infected patients would be detected, with the trade-off of conducting HIV testing on 8,008 patients. To identify all 50 infected patients, the figure suggests that one would need to screen nearly the entire population. The authors state as much, highlighting for the validation sample, "the top 3 risk groups represented 62.5%… of all patients diagnosed with HIV infection" (1, p 843), meaning that 37.5% of those infected would not be identified unless one also tested the lower-risk groups. The risk-score model appears to offer a very modest advantage over general HIV testing in the settings examined at the cost of obtaining extensive risk data to construct the score. This is aligned with a number of previous studies of targeted screening in clinical populations (3–5) and the CDC guidelines (2).

We have additional concerns about the populations and methods used. First, in selecting a model derivation population of individuals reporting to a sexually transmitted infection clinic for HIV testing and a validation population of individuals clinically and behaviorally indicated for HIV testing, the authors have partially conditioned their analytic data on risk factors included in the final model. This likely biases observed associations with HIV infection and may help to explain why some traditionally strong predictors of undiagnosed HIV infection (e.g., partner number, condom use) were not retained in the final model. Because populations at increased risk were used for the score development, it is also unclear how this model would perform for more general healthcare settings with patients who have a broader distribution of risks and for which the CDC guidelines were intended. Second, it is unclear how the authors ascertained whether HIV-positive individuals were previously undiagnosed. Substantial numbers of individuals who tested HIV-positive may have had a previous diagnosis, and this may be an important consideration because risk factors for failure to disclose a previous diagnosis may differ from risk factors for an undiagnosed HIV infection (6–8). Last, we question the validity of constructing a risk score based on the addition of model slope parameter estimates from a logistic regression model (Table 2). This method is typically reserved for linear regression, whereas in the context of a logistic regression model, these parameters contribute multiplicatively to risk (9).

Risk-score approaches to guide screening decisions are appealing, but in this case we feel that the data source limits the generalizability of the score to general populations. Even if it were generalizable, the results of the receiver operating characteristics curve indicate that a screening program, with higher costs required to identify patients for screening, would still require screening nearly everyone in a population to find those all of those living with HIV.

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LETTER TO THE EDITOR

Helicobacter pylori Infection Might Contribute to Esophageal Adenocarcinoma Progress in Subpopulations With Gastroesophageal Reflux Disease and Barrett's Esophagus

To the Editor,

Fischbach et al. [1] concluded that estimates for the effect of *Helicobacter pylori* (*H. pylori*) on Barrett's esophagus (BE) were heterogenous across studies; although overall *H. pylori*, and particularly *cag*A cytotoxin, tended to be protective for BE in most studies, *H. pylori* effect on BE varied by geographic location.

Barrett's esophagus is a complication of long-standing gastroesophageal reflux disease (GERD) and well-recognized premalignant condition playing a pivotal role in the development of esophageal adenocarcinoma (EA), the most common esophageal malignancy in Western countries with increasing faster incidence than any other cancer [2]; GERD plays a crucial role in the pathophysiology and the clinical identification of BE [2]. In this regard, our data show that *H. pylori* infection *(H. pylori*-I*)* is frequent in Greek patients with GERD and even with nonendoscopical reflux disease [2], and *H. pylori* eradication leads to better control of GERD symptoms and improves esophagitis [2,3]. Moreover, consistent associations with the Greek data were shown by others [3] also reporting improvement in reflux symptoms following *H. pylori* treatment. It is important to note that some other authors, usually prior supporters of the theory that *H. pylori* "protects" against GERD, relented their initial findings, claiming that *H. pylori* eradication does not cause or protect against GERD and, moreover, recommending *H. pylori* eradication in GERD [4]. Additionally, although epidemiologic studies do not suggest causality with *H. pylori*, however, such studies support our and others' findings; for instance, a large study (~21,000 cases) showed that the decrease in *H. pylori*-I parallels the decrease in peptic ulcer prevalence, and the increase in GERD and reappearance of GERD after *H. pylori* eradication is rare. Moreover, contrary to expectation, patients hospitalized with duodenal ulcers (61,548 cases), obviously attributed to *H. pylori*-I, had a significant 70% excess risk of EA. Much evidence further potentiates the concern that *H. pylori* is not "protective" against GERD [5] and its complications including BE and EA.

The interplay between *H. pylori* and host factors plays an important role in the pathogenesis of GERD. Specifically, *H. pylori* may contribute to GERD pathogenesis by several mechanisms including release of several mediators, cytokines, and nitric oxide, which may adversely affect the lower esophageal sphincter (LES); direct damage of the esophageal mucosa by bacterial products; increased production of prostaglandins that sensitize afferent nerves and reduce LES pressure; and augmented acidity (by gastrin release) that exacerbate GERD [3].

The authors considered some putative pathways involving *H. pylori* and a decreased risk of BE [1]. However, these pathways might represent the one BE pathogenic "coin's" side. Regarding the other alternative side, gastrin, induced by *H. pylori*-I, is an oncogenic growth factor contributing to esophageal, gastric, and colon carcinogenesis and, in particular, playing a potential causal effect on neoplastic progression in BE; gastrin stimulates proliferation via JAK2- and Akt-dependent NF-kappaB (NF-κB) activation in Barrett's EA cells, shows antiapoptotic activity through upregulation of Bcl-2 and survivin, and upregulates cyclooxygenase (COX)-2 expression [2]. In this regard, *H. pylori*-I activates NF-κB, an oxidant-sensitive transcription regulator of inducible expression of inflammatory genes such as COX-2, which regulates gastrointestinal cancer cell growth and proliferation. In particular, *H. pylori*-I induced NF-κB and COX-2 expression in esophageal epithelial cells, playing a role in inflammation associated with BE and tumorigenesis in the esophagus [2]; upon colonizing esophagus, *H. pylori* increases the severity of esophageal inflammation and the incidence of BE and EA [6]. Moreover, recent evidence indicates that 1, *H. pylori*-I prevalence is high in BE; 2, neither *H. pylori*-I nor *H. pylori*-I by CagA+ strains reduce the risk of BE in certain populations with high prevalence of *H. pylori*-I; 3, the expected incidence of EA with persistent *H. pylori*-I is higher than that of EA after eradication of infection [5]; *H. pylori*-I may affect specific molecular alterations (genetic instability, E-cadherin methylation, and monoclonal antibody Das-1) associated with the pathogenesis of BE; and 4, *H. pylori* induces

Ki-67 expression and increased esophageal expression of Ki-67 observed in BE patients compared with GERD controls. Ki-67 proliferation fraction increases significantly from normal squamous epithelium to BE, dysplasia, and EA [2]. Of note, the occurrence of nitrate-reducing *Campylobacter* species in patients with BE may imply that there is a link in the initiation, maintenance, or exacerbation of disease processes leading to EA development.

Therefore, *H. pylori* might be involved in the GERD-BE-EA sequence, at least in certain geographic ethnic subpopulations. These different data should provide a more detailed and overall information regarding the association between *H. pylori*-I and BE-related EA.

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Letter to the Editor

Helicobacter pylori infection and colorectal carcinoma: pathologic aspects

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To the Editor,

Fleming *et al.* (1), by reviewing the pathologic aspects of colorectal neoplasms, summarized the pathogenesis and molecular classification of colorectal carcinoma (CRC) including mainly molecular pathways and environmental factors. However, they did not mention the potential pathologic aspects of environmental factors involved in colorectal oncogenesis, particularly in sporadic CRC.

More than 95% of colorectal cancers are sporadic, also mentioned by the authors (1), without a significant hereditary risk. Geographic variation in the incidence of CRC is substantial with a higher incidence observed in the West. Environmental factors contribute considerably to this variation (2); the majority of the sporadic cancer is believed to be due to modification of mutation risk by other genetic and/or environmental factors. Dietary factors may influence the oncogenic process by modifying intestinal transit time, altering the flow and recycling of bile, or changing the intestinal bacterial flora composition. Numerous studies support a role for the gut microbiota in colorectal oncogenesis and the colonic microbiota drives the progression towards colorectal malignancy including generation of reactive metabolites and carcinogens, alterations in host carbohydrate expression and induction of chronic mucosal inflammation (3); long-term colonization of the colon by rogue commensal bacteria capable of inducing chronic DNA damage could contribute to sporadic CRC developement, thereby suggesting sporadic CRC as an infectious disease (4).

In this regard, *Helicobacter pylori* (*Hp*), a curved spiral gram-negative bacterium found in the gastric mucosa of a large proportion of humans worldwide (>50%), has been evaluated as a possible etiologic agent for CRC and recent data indicate that there is a serological association between *Hp* infection (*Hp*-I) and the risk of CRC, especially for left-sided and early-stage cancers (5). Moreover, *Hp* seropositive subjects are associated with a modest increase in the risk for colorectal adenoma, and since *Hp*-I can increase the risk especially of advanced adenomas, the medical community should take into account that a preventive strategy is needed, and, furthermore, elucidating the pathophysiological role of *Hp* in the development of CRC is highly warranted (6). However, as mentioned by the authors (5,6), the serologic measurement of infection status is less than perfect, thereby representing a specific limitation of their studies. Indeed, the serological test does not discriminate between current and past infections and, apart from past infection that may even be more relevant for oncogenesis, such a distinction is essential because only current *Hp*-I induces humoral and cellular immune responses that produce or perpetuate chronic inflammatory processes in gastrointestinal tract with potential oncogenic sequelae; many neoplasms including colorectal adenomas and cancers arise at the sites of chronic inflammation and infection (7-10).

Based on histology, the practical gold standard for *Hp*-I diagnosis, our own preliminary studies indicated *Hp* presence in malignant tissue in 34 of 41 (82.9%) patients with CRC (23 men, mean age 73.6±7.9 years) (11). Extending these preliminary data we currently included 50 patients (28 men, mean age 71.3±9.7 years) with CRC and 25 patients (13 men, mean age 72.8±10.1 years) with colonic polyps with the following results: *Hp* presence in malignant and polyp tissues of patients were observed in 84% and 64%, respectively, confirming our preliminary data (12). It is important to note that, apart from Cresyl fast violet staining mainly used to detect *Hp*, its presence was also documented by immunohistochemical method (using polyclonal rabbit anti-*Hp* antibody (dilution 1:50, DAKO, Athens, Greece) in adenoma and malignant colonic tissues. Specifically, in accordance with Hong *et al.* (6),

Hp progressive increased presence was observed in our patients with adenomas associated with mild (50%) and moderate/high-grade (80%) dysplasia; the latter lesions are frequently described as advanced adenomas. However, contrary to the authors' considerations (6), our series showed an increased *Hp* presence in left-sided (79%) than in the proximal colon (21%) adenomas; left-sided cancers were also observed in 70.7% of our patients, a finding also noticed by Zhang *et al.* (5), thereby suggesting that *Hp*-I might be associated with a rather relevant risk increase in the left CRC.

The multistep model of gastric cancer postulates that there is initially an inflammation, caused mainly by *Hp*-I, which can lead to the development of chronic active gastritis. In a subset of these patients, this inflammatory process leads to the development of atrophic gastritis, followed by intestinal metaplasia, dysplasia, and, ultimately, early and advanced gastric cancer (13). It is considered that all stages prior to the development of high-grade dysplasia are potentially reversible, although this is still controversial (13). Because, *Hp* also induces inflammatory changes in colonic mucosa (14), it would be reasonable to further speculate, in view of our data, that chronic inflammatory process induced by *Hp*-I in colonic mucosa may lead to adenoma - mild-moderate/high grade dysplasia - CRC development sequence. These findings may emphasize the need for *Hp* eradication to prevent the development of both colon and gastric cancer (13).

In addition, we found that presence of *Hp* in malignant colonic tissue was associated with Ki-67 oncogene increased expression in all tumor specimens and low expression in all adjacent tissue specimens (15). Moreover, p53 increased and low expression was observed in 72.5% and 100% of tumor and adjacent tissues specimens, respectively. Likewise, antiapoptotic Bcl-2 protein was observed in 60% and 9% of tissue specimens, respectively, whereas proapoptotic Bax protein was observed in 9% and 100% of tissue specimens, respectively (15). Therefore, *Hp* colonizing colonic neoplasm tissue seems to be associated with an increased cell proliferation and impaired apoptotic process in malignant tissue compared with normal adjacent colonic mucosa, thereby possibly contributing to colon normal mucosa-adenoma-cancer sequence (15). In this regard, *Hp*-induced gastrin as an oncogenic growth factor, shows antiapoptotic activity through the Bcl-2 upregulation and contributes to gastric and colon carcinogenesis through stimulation of mutagenic and tumorigenic cyclooxygenase-2 expression (16). Animal models suggested the mitogenic action of gastrin to be limited to the left colon, elevated gastrin levels are more pronounced in their associations with rectal than with colon cancer, and the relation between hypergastrinemia and colorectal adenomas confers an increased risk only for distal colon adenomas. These findings are consistent with and may explain our findings and Zhang *et al.* (5) findings of selective risk increase with respect to left-sided CRC and adenomas.

Experimental data indicate that *Hp*-I leads to development of chronic inflammation, hyperplasia, metaplasia, dysplasia and recruitment and accumulation of bone marrow-derived cells (BMDCs) which may contribute to tumor formation in animal models with *Hp*-induced chronic gastric inflammatory process (9,13). Because *Hp* similarly induces the mentioned inflammatory changes in colonic mucosa (14), it would be reasonable to further speculate that chronic *Hp*-I in humans also induces repopulation of the colon with BMDCs that might facilitate colon adenoma and cancer development and progression (9,13). In this regard, our own preliminary studies indicated increased expression of CD44 [a marker of human hematopoietic stem and progenitor cells and cancer stem cells (CSCs)] in malignant colonic tissue in 75.6% patients with CRC (11). Extending these preliminary data, increased expression of CD44 was observed in 78% and 16% of patients with cancers and polyps, respectively (12). We also obtained comparable data with gastric cancer (9,13). Therefore, these findings suggest the possible BMDCs and/or CSCs involvement in *Hp*-associated gastric cancer development and colon adenoma and cancer growth and/or progression (9,13).

However, larger-scale future studies are warranted to show that the BMDCs move into areas of the upper and lower gastrointestinal tract and/or CSCs might be induced in the context of *Hp* chronic injury or inflammation with potential long-term colon adenoma malignant consequences in *Hp*-positive subjects. Finally, it is important to know if the authors (1) considered relative pathologic aspects in their studies.

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REVIEW
Immuno-Gastroenterology 1:2, 104-107; October/November/December 2012; © 2012 STM Publishing

Helicobacter pylori and Immune Thrombocytopenic Purpura

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Summary

Helicobacter pylori (*H. pylori*) infection is a constantly growing subject of research since it was proven that several extra-intestinal diseases correlate to the tropism of pathogen in the gastric mucosa. The association between *H. pylori* and hematological disorders such as immune thrombocytopenic purpura (ITP), gastric mucosa-associated lymphoid tissue (MALT) lymphoma and iron deficiency anemia has been reported. In this paper, we review the literature regarding the role of *H. pylori* infection in ITP. Although the molecular mechanisms have not yet been fully understood, *H. pylori* eradication resulted in high response rates in ITP without major side effects.

Immunogastroenterology 2012; 1:104-107

Key words

Helicobacter pylori; immune thrombocytopenic purpura; eradication therapy; pathogenesis

Introduction

Immune thrombocytopenic purpura (ITP), an autoimmune disease caused by sensitization of platelets by auto-antibodies leading to increased platelet destruction mainly by spleen macrophages, has been associated with many infectious agents. Many published data refer to the pathogenesis of ITP whose etiology seems multifactorial. In addition to increased platelet destruction, some patients with ITP also have impaired platelet production. Recent studies also illustrate the possible implication of *Helicobacter pylori* (*H. pylori*) infection to the pathogenesis of ITP (**Fig. 1**). Eradication of the infection has been reported to produce an increase in platelet count in some studies, whereas other reports have failed to demonstrate such beneficial effects.[1-3] *H. pylori* infection is considered to be one of the most common human infectious diseases. Its prevalence is estimated to be 80% amongst the population in developing countries and 20-50% in the developed ones (>70% over the age of 50 years). *H. pylori* is a gram-negative micro-aerophilic spiral shaped bacterium which produces enzymes, toxins, and stimulates the outburst of various inflammatory mediators while colonizing gastric mucosa. Bacterial adhesion to the intestinal epithelium (intense tissue-type tropism) is a critical initial step in the pathogenesis of many enteric diseases. *H. pylori* is a duodenal pathogen that adheres to the gastric epithelium and causes gastritis and peptic ulceration. It is the most common extra-cellular micro-organism and it can rarely be found intra-cellularly.[4]

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The infectious elements of *H. pylori* are divided to those promoting colonization and those inducing the tissue type lesion.[5] The three main pathologies associated with *H. pylori* infection are: 1. duodenal and gastric ulcer; 2. gastric carcinoma; and 3. mucosa-associated lymphoid tissue (MALT) lymphoma. Moreover, according to recent data, *H. pylori* infection has been also implicated in the pathogenesis of ITP among other auto-immune diseases, such as auto-immune pancreatitis, Guillain-Barré syndrome and multiple sclerosis.[6-9]

The reference standard for the diagnosis of *H. pylori* infection is endoscopic biopsy and histopathologic confirmation of the organism, which is often impractical. Noninvasive tests include serum antibody titers, the ^{13}C-urea breath test and stool testing with either polymerase chain reaction or antigen enzyme immunoassay. The ^{13}C-urea breath test and antigen detection in stools are considered to be the most accurate, with both sensitivity and specificity in the range of 90-95%. Serologic detection may be used but is less sensitive and less specific than the other tests. Furthermore, the test may produce false positive results after intravenous immunoglobulin therapy (IVIg).[3,4,9]

In this paper, we review the published peer-reviewed literature regarding the role of *H. pylori* infection in ITP.

Clinical data

The prevalence of *H. pylori* infection in adult ITP patients has been systematically reviewed and was not found different from that reported in the general healthy population matched for age and geographical area. The detection method in these studies was the ^{13}C-urea breath test. The prevalence of *H. pylori* infection in adult ITP patients is 22% in the North American Caucasian population, 29% in the white French population. Furthermore, it is nearly 50% in Italy, greater than 70% in Japan, 90.6% in

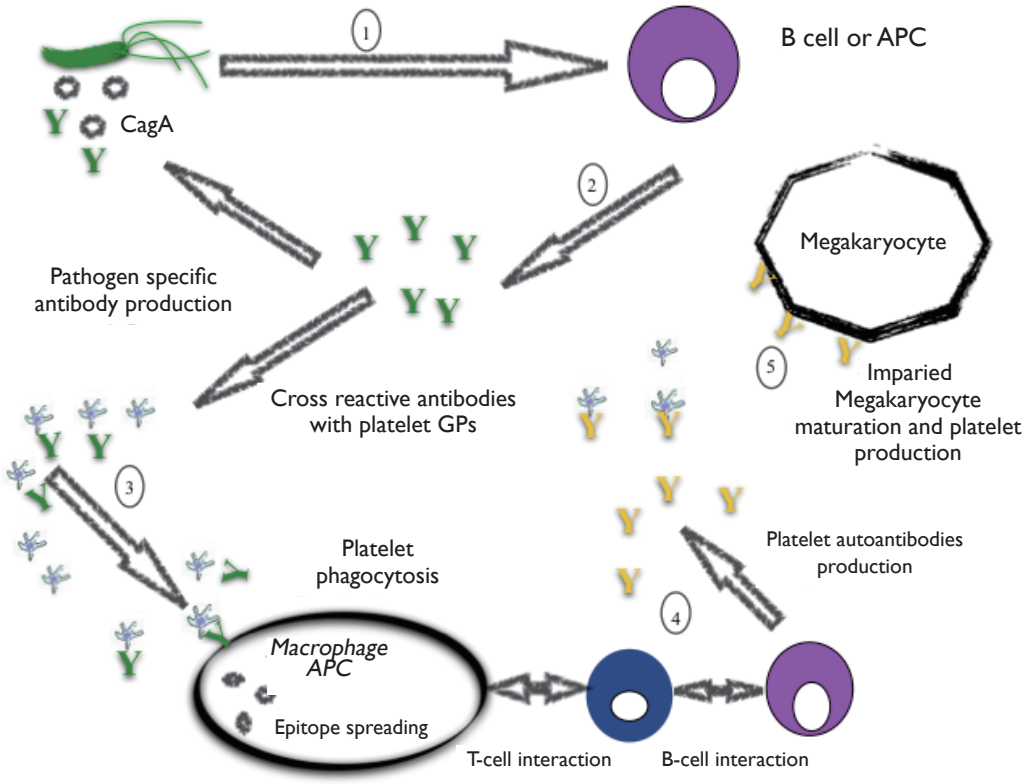

Figure 1. Mechanism of immune thrombocytopenia induced by *Helicobacter pylori* (*H. pylori*) through molecular mimicry. *H. pylori* CagA protein provokes B-lymphocytes to produce pathogen specific antibodies that cross-react with platelet surface glycoprotein agents. Opsonized platelets are taken up and destroyed by macrophages predominantly in the spleen. They also bind bone marrow megakaryocytes, thereby impairing megakaryocyte maturation and platelet production. *CagA*, cytotoxin associated geneA; APC, antigen presenting cells; GPs, glycoproteins.

Colombia.[9,10]

It is of great importance that in the first report of the association between *H. pylori* infection and ITP, only the beneficial action of omeprazole in patients with chronic ITP was mentioned.[11] Gasbarrini et al. were the first to describe a platelet count improvement in 8 of 11 patients with ITP following eradication of *H. pylori* and resolution of platelet autoantibodies in 6 of these 8 patients.[12] Furthermore, eradication treatment used for *H. pylori* with proton pump inhibitors (omeprazole 20 mg b.i.d.) in combination with amoxicillin (1000 mg b.i.d.) and clarithromycin (500 mg b.i.d.) for 7-14 days seems beneficial for the majority of patients and increased the absolute number of platelets. After completion of the antibiotic course, eradication of the offending bacteria must be ensured by repeating the breath test or stool test. Several systematic reviews have examined the diagnosis and the efficacy of eradication of *H. pylori*.[10,13-15] A systematic analysis of 25 published studies, in 800 patients, mainly in Italy and Japan, with platelet levels usually >30*10^9/L, showed successful eradication therapy in 72% of the patients and improvement in thrombocytopenia in 75-80% of them, which means overall improvement in 50-55% of the patients.[15] In this analysis, there were few patients with platelets <30*10^9/L. Eradication treatment was well tolerated with mild side effects. The commonest were abdominal pain and diarrhea but only a minority of patients stopped the therapy.[15] It is worth mentioning that favorable results in thrombocytopenia after eradication therapy for *H. pylori* have been reported also in *H. pylori*-negative patients, in patients resistant in immunoglobulin treatment and splenectomy and in patients with severe thrombocytopenia.[16,17] This finding could be explained in several ways, including an immunomodulatory effect of macrolides separate from the bacteriostatic effect. However, these findings were not proven by all studies.[10,18] The role of age in achieving a favorable response is very controversial. The prevalence of *H. pylori* infection in the general population increases with age. In a recent study, it is reported 100% response, in patients above 60 years of age and 22% in patients less than 50 years, while in other studies age had no prognostic value in the response rate.[16,17,19]

The sustainability of the response appeared satisfactory, as in 8 years of follow up, platelet counts of the 11 previous responders remained stable indicating complete response.[20] A similar result was also observed in another study, as in 5 years of follow up 23/24 of patients with successful eradication of *H. pylori*, retained the favorable response and only one case relapsed (**Table 1**).[20-23]

Pathogenesis

The actual pathogenetic mechanisms between *H. pylori* and ITP have not been clarified yet. There are many speculations about pathogenesis of ITP in patients with *H. pylori* infection.

Table 1. Long term platelet responses to *Helicobacter pylori* eradication in patients with immune thrombocytopenic purpura

Reference	No of patients positive-treated	Country	Response	Follow-up
Kikuchi T et al.[20]	11	Japan (2011)	100% CR	8 years
Jackson SC et al.[22]	4	Canada (2008)	75% Response	48 months
Emilia G et al.[21]	34	Italy (2007)	68% Response	60 months
Satake M at al.[23]	23	Japan (2007)	100% CR	25.8 months.

It is suggested that active *H. pylori* infection triggers humoral and cellular immune responses, which probably induce *H. pylori*-associated diseases including ITP through molecular mimicry. Furthermore, many data indicate that the effect of eradication treatment for *H. pylori* probably depends on genetic factors of the host, strains of the bacterium or existing ethnic factors. According to these data, *H. pylori* infection influences the pathophysiology of ITP through various mechanisms.[17,24-29]

According to the most predominant mechanism, antibodies against virulent factors of *H. pylori*, such as *CagA* and *VagA*, cross-react against various platelet glycoprotein antigens. These two proteins are often co-expressed and the second one possesses cytotoxic properties. The genomic complex of the CagA protein of *H. pylori* contains 40 genes and one of these participates in the formation of the expression complex. This complex is absent in asymptomatic carriers of the bacterium. The pathogenetic role of CagA protein in ITP has been highlighted in two studies. In the first one, a decline in platelet-associated immunoglobulin G in ITP patients after the eradication of *H. pylori* infection as well as the existence of a molecular mimicry between those antibodies and the CagA protein has been shown.[17] In the second one, it was proven that the anti-CagA antibodies cross-react with peptides which were expressed in the platelets of ITP patients.[25] Other putative targets of molecular mimicry are Lewis (Le) antigens, expressed by *H. pylori* in a strain-specific manner. Le antigens are adsorbed to platelets and might serve as targets for anti-Le antibodies in patients with an appropriate genetic background.[26] Some bacteria activate platelets, increasing the binding affinity of von Willebrand factor to platelet membrane glycoprotein Ib (GPIb), thereby increasing the rate of platelet destruction. The variability in the effectiveness of eradication therapy for *H. pylori* could be attributed to the different expression of CagA protein which was found to have increased expression in Japanese patients.[17,28]

Another mechanism, which might explain the failure of eradication therapy and the heterogeneity of the responses, involves the installation of immunologic memory in T- and B-cell auto-reactive clones. Failure of correction of thrombocytopenia by decreasing the immunologic burden of *H. pylori* means development of auto-reactive clones, independent of the presence of bacteria which change the balance between activating and de-activating Fcγ receptor of monocytes.[24]

Furthermore, it seems that host genetic factors influence the pathogenesis of the disease. Patients with *H. pylori* infection and ITP have different HLA antigen profile compared with patients without ITP.[16] The importance of genetic factors emerged from the results of an Italian study, indicating that the ITP patients with *H. pylori* infection had a lower frequency of DRB1*03 and higher frequencies of DRB1*11, DRB1*14, and DQB1*03 relative to *H. pylori*–negative cases. Moreover, an HLA-DQB1*03 pattern was associated with a higher probability of platelet response to eradication treatment.[29]

The genomic polymorphism of cytokines could possibly be involved in the pathogenesis. Persistence of T helper (Th)-1 immunologic response after eradication treatment is associated with poor responses to thrombocytopenia.[27,28]

It was recently found that patients with *H. pylori* promote P-selectin expression in the presence of anti IgG against *H. pylori*, which increases the connection of *H. pylori* to platelets. P-selectin expression is followed by apoptotic messages that could influence platelet destruction during *H. pylori* infection.[30]

In conclusion, among patients with *H. pylori*-associated ITP, a significant percentage achieves lasting remission after eradication of *H. pylori*. This fact confirms the existence of an association between *H. pylori* and ITP. The pathogenesis of ITP-associated with *H. pylori* is still not well-defined, but it seems that genetic host factors and bacterial factors may both play important roles. These factors may also account for the variability in the response to eradication treatment observed in ITP *H. pylori*-infected patients.[13]

Although empiric *H. pylori* eradication therapy in ITP is appealing because of its simplicity, less toxicity and low costs compared to standard (steroids or splenectomy or immunosuppressive drugs) or new (thrombopoietic agents, romiplostim or eltrombopag) ITP therapy, the literature suggests that treatment should be reserved for *H. pylori*-positive patients only. High response rates and high prevalence of the infection in certain countries like Japan support routine screening for *H. pylori* in those regions. In countries where prevalence is low, screening may be justified since some patients may respond to treatment. Further randomized trials are needed to fully ascertain the role of *H. pylori* in ITP and for longer duration of follow up to assess the rate of relapse among the recovered cases and identify factors that may assist in selecting ITP patients who are more likely to respond to therapy.[31,32]

Conflicts of interest

The authors declared no conflicts of interest.

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