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Alimentary Tract

Diagnostic value of rapid urease test and urea breath test for *Helicobacter pylori* detection in patients with Billroth II gastrectomy: A prospective controlled trial

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Abstract

Aim. The aim of this work was to assess the reliability of rapid urease test (RUT) and urea breath test (UBT) for detecting *Helicobacter pylori* (*H. pylori*) in patients with Billroth II (BII) gastrectomy, using histology as reference.

Methods. In this prospective controlled study, 31 consecutive patients with BII gastrectomy and 73 controls who had an indication for endoscopy were included. Their *H. pylori* status was assessed with biopsies for histology, RUT and UBT. Histology served as the gold standard. Only the biopsies from the gastric fundus were evaluated. Specificity, sensitivity, positive and negative predictive value, degree of agreement and *k*-statistics were used.

Results. RUT and UBT for detecting *H. pylori* in the control group had excellent agreement [97%, kappa (k) = 0.94 and 99%, k = 0.97 respectively] with biopsies. In BII patients, RUT from fundic biopsies had very good agreement (87%, k = 0.74) compared to histology from fundic biopsies, whereas the UBT was unreliable (agreement: 71%, k = 0.41) compared to histology.

Conclusion. The RUT from fundic biopsies in BII patients is a reliable test for *H. pylori* detection, whereas the UBT is unreliable.
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Keywords: Billroth II; *Helicobacter pylori*; Kappa agreement; Rapid urease test; Sensitivity; Specificity; Urea breath test

1. Introduction

The clinical significance of *H. pylori* in the intact stomach is well known. However, its role in gastrectomized patients for the development of a future benign or malignant disease remains controversial. Current consensus strongly recommends its eradication after gastrectomy for gastric cancer [1].

It seems that Billroth II (BII) gastrectomy carries a higher risk for gastric cancer development in the remnant stomach many years after the operation, even when this is performed to manage benign disease [2,3]. *H. pylori* detection, as a possible additional carcinogenic factor in such patients, is crucial. Different tests for *H. pylori* detection in the intact stomach are available, but biopsies for histological examination seem to be the only reliable test in patients with BII gastrectomy. It is of great importance that the exact site for biopsies in these patients has not been yet standardized, and no prospective controlled trials have addressed this issue.

In common clinical practice, histology, rapid urease test (RUT) and urea breath test (UBT) are often the most widely available tests for *H. pylori* detection. Their value as screening tests, in patients with BII gastrectomy who are going to be gastroscoped, has not been examined in a systematic and controlled manner. The present study aims to investigate the reliability of the above tests in this population.

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2. Patients and methods

2.1. Patients and controls

This is an one centre-conducted study including consecutive patients with confirmed BII gastrectomy and controls without gastrectomy.

The BII patients who participated were either symptomatic (epigastric pain and/or vomiting, anaemia, melaena) or asymptomatic (referred for endoscopy as a follow-up examination or for anaemia). A surgical report was obtained from all the BII patients, for the confirmation of the type of operation. The consecutive outpatients who served as controls during the same period of time had an indication for endoscopic investigation either for upper abdominal symptoms or for iron deficiency anaemia. For each BII patient, the next two consecutive patients with the same indications for endoscopy as for BII patients were recruited as controls.

As the aim of the study was the investigation for *H. pylori* infection, exclusion criteria were restricted to those that could confound *H. pylori* detection. Per protocol, patients who reported proton pump inhibitors, H2-receptor antagonists or antibiotic intake for less than 8 weeks were excluded. For ethical reasons, simple antacids were allowed for use until 4 weeks, spasmolytics until 1 week and paracetamol until 3–4 days before endoscopy. Patients with absolute contraindications for endoscopy or on anticoagulants that could not be stopped were excluded from the study.

The Hospital Ethics Committee approved the study and all patients signed a consent form after a full explanation of the procedure and their participation in the trial.

2.2. Endoscopy

Endoscopy was performed by a single, experienced endoscopist (A.B.A.), after a conscious sedation with 5–20 mg of diazepam iv. Beyond the other findings, the gastric remnant condition was assessed according to the Hisanori A et al. classification [4].

2.3. H. pylori assessment

H. pylori detection tests were performed as described in the following text.

2.3.1. Billroth II patients

Biopsies. Six biopsies were taken with an ordinary biopsy forceps from the mucosa of the gastric fundus and were examined by a single experienced pathologist (T.D.) who was blinded to the patients' status. H&E stain and the modified Giemsa stain were used for *H. pylori* detection in gastric biopsies. Those patients who were found positive in any specimen were considered *H. pylori* positive. The density of the *H. pylori* organisms was, per protocol, not measured.

Two additional biopsies for RUT were taken from the gastric fundus as well, and examined in the same patch dot by Pronto dry test (Medical Instruments Corp., Solothurn, Switzerland). The result was evaluated after 30, 60 and 120 min thereafter, according to the manufacture's instructions.

Additional biopsies were taken from any suspicious lesion found during the endoscopy, from the anastomosis and from sites proposed by Safatle-Ribeiro et al. [5] but none was evaluated for the presence of *H. pylori*.

Urea breath test. UBT was done 4–6 h before gastroscopy, after an overnight period of fasting, with the only commercially available test (INFAI, Institut für biomedizinische Analytik & NMR-Imaging GmbH, Bochum), according to the manufacturer's instructions: 200 ml of commercially available orange juice (100%, without sugar) was given orally after two basal breath samples in 10-ml-plastic vacutainers had been taken. After the ingestion of the orange juice, 75 mg ^{13}C-urea powder dissolved in 30 ml water was administered and the patients remained recumbent till the next sampling.

Breath samples were collected 30 min after the ingestion of the ^{13}C-urea. The breath samples were analyzed by mass spectrometry and $^{13}CO_2/^{12}CO_2$ ratios in breath were standardized with respect to PDB (Pee Dee Beliminate). *H. pylori* infection was deemed present if the difference in $^{13}C/^{12}C$ of baseline-value and 30-min-value exceeded 4.0‰.

2.3.2. Controls

Biopsies. Six biopsies with an ordinary biopsy forceps were taken from normal-appearing mucosa of the gastric prepyloric region, incissura and lower gastric body (two from each of the above-mentioned parts) and were examined and evaluated as in the BII group.

Additional biopsies were taken from any suspicious lesion found during the endoscopy. Two additional biopsies were taken from the antrum for RUT examination.

Urea breath test. The urea breath test was performed in the same way as in patients with BII gastrectomy. Also, the patients stayed recumbent for 30 min, as those with BII gastrectomy, in order to establish the same conditions.

2.4. Statistics

The sensitivity, specificity and positive and negative predictive value of the different tested methods (RUT and UBT) in both groups (BII and control group) were calculated by taking biopsy as the reference diagnostic method. The kappa statistic was used to determine the level of agreement in diagnosis among tested methods and the reference method. Statistical analysis was performed using the Minitab statistical software (release 13.31; Minitab, Inc., State College, PA). A probability value of $p < 0.05$ was considered statistically significant.

Table 1
Indications for upper gastrointestinal endoscopy

Indication	Control group ($n = 73$)	Billroth II group ($n = 31$)
Epigastric pain, vomiting, heartburn or combinations of the above	55	11
Anaemia	18	9
Gastrointestinal bleeding	0	4
Investigation for metastatic cancer	0	1
Follow-up (gastric malignancy)	0	6

3. Results

This study was performed between July 2004 and April 2007 and included 31 consecutive patients with confirmed BII gastrectomy and 73 controls without gastrectomy. The median age of BII patients was 71 years (range 28–86) and of controls 63 (range 29–86), whereas the male/female ratio was 23/8 and 49/24, respectively. Four BII patients presented with melaena and none of them had active bleeding during the urgent endoscopy or any exclusion criteria for the study. The indications for endoscopy are shown in Table 1. The BII gastrectomy was performed for a benign cause in 23 of 31 patients (benign ulcer, perforation, bleeding) and in 8 of 31 for gastric cancer. The median time elapsed after surgery was 17 years (range 1–45). In the majority of participants, *H. pylori* status before gastrectomy was not known, as it was mainly not searched for at that time. The endoscopic findings in both controls and BII participants are shown in Table 2.

In the control group, taking into account that the reference method was biopsies for histological examination for *H. pylori*, both the RUT and UBT had excellent agreement (97%, $k = 0.94$ and 99%, $k = 0.97$, respectively) compared with histology (Table 3).

The BII patients, in whom fundic biopsies for histology served as the reference method, the RUT was found to be acceptable for *H. pylori* detection test [6], provided that the biopsy specimens were taken from the fundic mucosa as well (agreement 87%, $k = 0.74$) (Tables 3 and 4).

The UBT (the commercially available INFAI breath test), even done in the way described above (in recumbent position), was an unreliable test for *H. pylori* screening test, in patients with BII gastrectomy (agreement 71%, $k = 0.41$) (Tables 3 and 4).

Overall, 48.38% (15/31) of patients with BII gastrectomy, a rather high percentage of the studied population, were infected with *H. pylori*, a number close to our control group (63%) (46/73) (Table 4).

Table 2
Endoscopic findings in participants

Control group ($n = 73$)		Billroth II group ($n = 31$)	
Normal-appearing mucosa	53	Remnant gastritis, Grade 0[a]	10
Gastric erosions	3	Remnant gastritis, Grade 1[b]	4
Peptic ulcer	6	Remnant gastritis, Grade 2[c]	2
Oesophagitis	10	Remnant gastritis, Grade 3[d]	11
Angiodysplasias	1	Anastomotic ulcer	3
		Anastomotic mass	1

[a] No redness of remnant gastric mucosa.
[b] Mild redness around anastomosis.
[c] Comb-shaped marked redness in the greater curvature on the oral side of the anastomosis.
[d] Diffuse severe redness and marked edema (grading according to [4]).

Diffuse severe redness and marked edema [4] (also named alkaline gastropathy) during endoscopy seems not to foresee to the presence of *H. pylori*, as this entity assimilates to gastritis grade 3, according to the Hisanori et al. classification [4]. In our BII group, with this endoscopic view, 7 of 11 were *H. pylori* positive and 4 of 11 negative. Our sample size was quite low for any further remarks.

4. Discussion

The aim of our study was to assess the diagnostic ability of the most commonly performed tests for *H. pylori* detection in patients with BII gastrectomy who are going to be investigated by an upper gastrointestinal endoscopy. This study is the first one that included a group of controls, prospectively studied, who also needed an upper gastrointestinal endoscopy, in order to evaluate the different tests in

Table 3
Comparison of the diagnostic value of RUT and UBT between Billroth II and control group of participants using biopsy as reference method

Group	Tested method	Sensitivity (%)	Specificity (%)	Predictive value (%)		Agreement (%)	Kappa statistic
				Positive	Negative		
BII	RUT	72 (47–88)	97 (77–100)	96 (70–100)	79 (57–91)	87	0.74 (0.51–0.97)
Control	RUT	95 (84–98)	98 (85–100)	99 (90–100)	92 (77–97)	97	0.94 (0.86–1.00)
BII	UBT	41 (21–64)	97 (77–100)	93 (56–99)	64 (44–79)	71	0.41 (0.14–0.67)
Control	UBT	99 (90–100)	95 (82–99)	97 (87–99)	98 (86–100)	99	0.97 (0.92–1.00)

BII, Billroth II; RUT, rapid urease test; UBT, urea breath test. Values are presented as means (95% confidence intervals).

Table 4
Tests for *H. pylori* status

	Control group ($n = 73$)		Billroth II group ($n = 31$)	
	Histology, *H. pylori* positive ($n = 46$)	Histology, *H. pylori* negative ($n = 27$)	Histology, *H. pylori* positive ($n = 15$)	Histology, *H. pylori* negative ($n = 16$)
RUT positive	44	0	11	0
RUT negative	2	27	4	16
UBT positive	43	1	6	0
UBT negative	0	29	9	16

RUT, rapid urease test; UBT, urea breath test.

BII patients under the same clinical conditions instead of using historical controls. This study has group homogeneity (does not include operated stomachs with different types of surgery). Patients with a BII operation carry a potential long-term risk for gastric remnant cancer development after the operation [2,3,5,7], even if the operation is performed for a benign condition.

It is still unclear whether only *H. pylori* plays a harmful role in the gastric remnant of the BII patients. Additional factors might play a significant role in gastric carcinogenesis in resected stomachs [2,3,7]. Consensus bodies strongly recommend an eradication strategy in the early postoperative period, if the operated stomach had harboured cancer [1]. No consensus recommendations exist for those operated for a benign disease, although it seems that it is recommended – as is their endoscopic surveillance [5] – especially for those who were operated early in their life.

In intact stomachs, the sites for biopsies for *H. pylori* detection are well known. In patients with BII gastrectomy, these sites have not been standardized yet, and so the gastric site from which they are taken for histology and RUT in BII patients seem to be still unclear. No prospective controlled study using tools for explanation of its statistical analysis exists on this field. Biopsy in the vast majority of studies remains the gold standard during endoscopy, either in intact or in gastrectomized stomach. To our knowledge, only one study [8] speculated that biopsy is an unreliable test, and it was based on *H. pylori* detection in stool. However, this study did not compare biopsies directly with stool *H. pylori* detection, but was based on the statement of a previous study in intact stomachs in which Gram's stain was used for *H. pylori* detection [9]. So it seems that there is rather inadequate evidence to exclude biopsies as a reference method in gastrectomized patients yet.

Because of the study of Shilling et al. [10], we chose the fundic mucosa in BII patients for taking biopsies, either for histology or for RUT. According to our results, fundic biopsies for RUT can be used as a good and acceptable screening test [6,11] (although not so excellent as in controls), provided that the biopsy specimen is taken from the gastric fundus, as our study indicates. This issue has not been taken into account in other studies.

The unreliability of the test in other studies comes probably from the following facts: (a) the biopsies were corporeal [12]; (b) the specimen for CLO (alternative RUT) test comes from an unreported site [13], but surely not from the fundus; and (c) the explanation of the unreliability of the test is not so firm.

The UBT appears to be a poor diagnostic and screening test in patients with BII gastrectomy, even in recumbent position, despite studies showing the contrary [14]. It appears unreliable because of rapid gastric emptying and/or enterogastric alkaline refux [15], at least in the cut off 4.0‰ of the commercially available INFAI test.

In our study, *H. pylori*'s presence in patients with BII gastrectomy is high (∼35%) compared with the group of controls (44/73; ∼60%) and with a 50% presence estimated in the general population in this country [16].

According to our results, fundic biopsies are crucial either for histology or for RUT, as fundus seems to be the niche of the *H. pylori*. We emphasize the fact that all the study's participants (BII patients and controls) had an indication for gastroscopy, and so the need for a gold standard (biopsies) was necessary for designing the study. Additional non-invasive tests were not used in this study. Culture is a time-consuming method, not widely available and carries a low sensitivity in intact stomachs [17]. Serology is also inferior to UBT and stool antigen test in intact stomachs [17]. Stool antigen test seems promising, especially the one that uses monoclonal reactant [18–21]. It was unavailable for our study; so it needs further evaluation in patients with BII gastrectomy, as it is simple in the common clinical practice.

In this study, the endoscopic appearance of the gastric mucosa (grade 3 gastritis) [4], also named alkaline gastropathy, was not helpful to foresee the presence or absence of *H. pylori*.

In conclusion, it seems that a change in the theoretical basis for the selection of the site for biopsies in patients with BII gastrectomy is needed. Fundic biopsies for histology and rapid urease tests are mandatory for *H. pylori* presence. Furthermore, fundic RUT seems a reliable additional screening test for these patients, although it has a lower sensitivity than controls. It is worthwhile to try this test whenever an endoscopy is needed in patients with BII gastrectomy, as a final medical decision and recommendation can follow immediately after the endoscopy. If positive biopsies taken only for *H. pylori* detection by histology can be discarded, patients can be protected from anxiety and loss of time.

Practice points

- ^{13}C-Urea breath test is unreliable for screening patients with BII gastrectomy, for the detection of *H. pylori*, even if the patients are placed in recumbent position.
- As fundic biopsies serve as the gold standard for *H. pylori* detection in patients with BII gastrectomy who have indication for an upper gastrointestinal endoscopy, RUT from the same site has very good agreement with them.
- The very good agreement of the fundic RUT with histology from fundic biopsies allows the former to be considered as a good, quick screening test for the patients with BII gastrectomy who undergo upper gastrointestinal endoscopy, although it is inferior to the RUT taken from controls with intact stomachs and have the same indications for endoscopy with the BII patients.

BII, Billroth II; RUT, rapid urease test.

Research agenda

- Stool antigen test should be compared to fundic biopsies in patients with a BII gastrectomy, even if these patients need an upper gastrointestinal endoscopy, in order to find out if it can be used as the gold standard for *H. pylori* detection as a quick test in routine clinical practice.
- Fundic mucosa should be the site of choice for other non-routine tests and studies for *H. pylori* in patients with BII gastrectomy.

BII, Billroth II.

Conflict of interest statement

None declared.

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From the "little brain" gastrointestinal infection to the "big brain" neuroinflammation: A proposed fast axonal transport pathway involved in multiple sclerosis

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SUMMARY

The human central nervous system (CNS) is targeted by different pathogens which, apart from pathogens' intranasal inoculation or trafficking into the brain through infected blood cells, may use a distinct pathway to bypass the blood–brain barrier by using the gastrointestinal tract (GIT) retrograde axonal transport through sensory or motor fibres. The recent findings regarding the enteric nervous system (often called the "little brain") similarities with CNS and GIT axonal transport of infections resulting in CNS neuroinflammation are mainly reviewed in this article. We herein propose that the GIT is the vulnerable area through which pathogens (such as *Helicobacter pylori*) may influence the brain and induce multiple sclerosis pathologies, mainly via the fast axonal transport by the afferent neurones connecting the GIT to brain.

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Infections and multiple sclerosis

A common characteristic of many neurodegenerative disorders of the central nervous system (CNS) is neuroinflammation, marked by augmented numbers of activated and primed microglia, increased steady-state levels of inflammatory cytokines and decreases in anti-inflammatory molecules. These conditions sensitise the brain to produce an exaggerated response to the presence of an immune stimulus in the periphery or following exposure to a stressor [1,2]; neurodegenerative brain disorders such as multiple sclerosis (MS), Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis involve neuroinflammatory reactions [3–5].

Specifically, MS is a chronic, unpredictable inflammatory demyelinating disease in the CNS; it affects more than two million people worldwide and has been recognised as the leading cause of neurological disability causing chronic paralysis and immersing socio-economic problems among young adults [6]. The aetiology of MS is an elusive field due to the lack of the standard cause–effect relationship model to explain a disease. Despite the enormous hard works made in MS research, the pathogenic mechanisms involved in the inflammatory and degenerative process of MS are still unclear. In fact, current data suggest that MS is a multi-factorial disease, where an infective agent on a genetically permissive host can result in neuroinflammation, demyelination, and ultimately in neurological damage with dire consequences for the patients. While genetic susceptibility explains the familial clustering of MS and the sharp decline in risk with increasing genetic distance, it cannot completely explain the geographical variations in the MS frequency and the changes in risk that occur with migration, which support the action of strong environmental factors. Among these, infections are emerging as the most consistent predictors of MS risk [7]; genes and infections by pathogens might act synergistically to trigger the disease [8]. In this respect, the possibility that microorganisms can cause MS has recently been addressed: Epstein-Barr virus, human herpes virus 6, *Chlamydia pneumoniae* (*C.* pneumoniae), and possibly *Helicobacter pylori* (*Hp*) by eliciting inflammation may cause the neurological damage that results in MS [5,8–12]. It has been suggested that the communicable factor is acquired in early adolescence [13]. Although the early events underlying MS remain uncertain, active inflammation may determine the initial phase of the disease. The infectious agents may exist at the origin of MS and other autoimmune diseases [14,15]; infection-induced molecular mimicry can induce autoimmune disorders including an early onset of the demyelinating disease associated with activation of CD4+ T cells [14].

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While demyelination might be a secondary phenomenon in the acute lesions of MS and the first visible event appears to be the apoptotic death of oligodendrocytes [16], active plaques occur early during the disease and are characterised by the presence of mononuclear cells, including T- and B-lymphocytes and macrophages in brain perivascular spaces. Despite the fact that no single causal agent or event has yet been identified, a long-favoured hypothesis in MS pathology is largely attributed to autoreactive effector T cells generated in the periphery that penetrate the blood–brain barrier (BBB) and become activated within the CNS [17,18]. Importantly, as autoreactive T cells are present in the blood of MS patients, other regulatory mechanisms exist to prevent autoreactive T cells from causing immune disorders. In this respect, the function of T regulatory (Treg) cells which play a key role in the control of self-antigen-reactive T cells and the induction of peripheral tolerance may be diminished in MS and may be correlated with impaired inhibitory activity [18]. As a result, autoreactive CD4+ and CD8+ T cells have been found to invade and clonally expand in inflammatory CNS plaques in MS [19]. Of note, CD4+ effector T cells are categorised into three subsets: T-helper type 1 (Th1), Th2, and Th17 cells; the latter have also been involved in the pathophysiology of MD [20]. Specifically, the interaction of activated CD4+ T cells with microglia led to a pro-inflammatory Th1 response with a Th1-type cytokine expression profile involved in the pathogenesis of apoptotic neuronal cell death in MS; secretion of substantial levels of pro-inflammatory Th1-type cytokine tumour necrosis factor (TNF)-α leads to TNF-α-related apoptotic neuronal cell death in MS [21]. Notably, apoptotic rather than necrotic microglia-associated nerve cell death appears as likely to underlie a number of common neurological conditions including MS, AD, PD, and glaucoma ('ocular' AD) [3,22]. MS is crucially dependent on activation of pro-inflammatory Th1 T cells by antigen-presenting cells, resistance of T cells to Fas-mediated apoptosis is involved in its exacerbation, and auto-aggressive Th1 cells can be adoptively transferred to non-diseased recipient mice that subsequently develop disease [23].

The activated encephalitogenic immune effectors (CD4+, CD8+ T cells, B cells, and macrophages) express surface molecules that allow them to penetrate the BBB and enter the CNS [24]. However, the explanation of the initial process of breakdown of normally tight BBB and the association of cellular infiltration into the CNS remains unanswered. In this regard, a series of factors have been implicated in inducing BBB disruption, including inflammatory mediators [e.g., cytokines and chemokines induced by *Hp* infection (*Hp*-I)] and oxidative stress [25,26]. *Hp* could indirectly affect the brain and other target organs, e.g., the heart, through the release of numerous cytokines such as TNF-α acting at distance; TNF-α is involved in BBB disruption through a mechanism involving matrix metalloproteinases upregulation [27]. In addition, *Hp*-induced cytotoxin VacA exhibits chemotactic activities to the bone marrow-derived mast cells (BMD-MCs) and induces BMD-MCs to produce pro-inflammatory cytokines including TNF-α [28]; BMD-MCs reside adjacent to blood and lymphatic channels, mainly under epithelial surfaces including the BBB and gastrointestinal tract (GIT) [29]. MCs can be stimulated by corticotropin-releasing hormone, secreted under stress, to release mediators including histamine, interleukin (IL)-8, tryptase, and vascular endothelial growth factor, which disrupt the BBB [30]. Therefore, regarding the pathogens' access the CNS, apart from pathogens' intranasal inoculation, the influx of activated monocytes infected with pathogens such as *C. pneumoniae* through the disrupted BBB in the brain could lead to the development of degenerative diseases [31].

In this study we propose that the GIT is the vulnerable area through which pathogens (such as *Hp*) influence the brain and induce CNS neuroinflammation, via another rational pathway; inflammatory GIT reactions may access the brain and induce degenerative pathologies, mainly via axonal transport by the afferent neurones connecting the GIT to the brain.

From the periphery to brain

Gastrointestinal tract

Inflammation in the brain might initiate from the periphery and relative data suggest that peripheral conditions powerfully influence processes in the brain relevant to MS. Indeed, systemic infections influence CNS function, and microbial invasion and traversal of the BBB is a prerequisite for CNS infections. Pathogens can cross the BBB transcellularly, paracellularly, and/or in infected phagocytes (the so-called Trojan-horse mechanism). Subsequently, pathogens can induce BBB dysfunction, including increased permeability, pleocytosis, and brain pathologies [32,33]. Notably, sickness behaviour appears to be the expression of the adaptive reorganisation of the host priorities during an infectious episode. This process is triggered by pro-inflammatory cytokines (i.e., IL-1β, IL-6, IL-8, and TNF) produced by peripheral phagocytic cells in contact with invading microorganisms. The peripheral immune message is relayed to the brain through a fast neural pathway and a slower humoral pathway, leading to the expression of pro-inflammatory cytokines in macrophage-like cells and microglia in the brain [32].

An alternative route of entry for pathogens into the CNS is through the nasal olfactory pathways [31]. Because, *C. pneumoniae,* being a causal factor of MS [34], readily infects epithelial cells and has direct access to the olfactory neuroepithelium of the nasal olfactory system, this pathway of infection would seem likely, given that *C. pneumoniae* is a respiratory pathogen. Subsequently, infection, inflammation, and/or damage of the olfactory bulbs could result in brain damage [31].

The nasal olfactory pathway is connected directly and the blood pathway is connected indirectly with GIT, a susceptible area by which pathogens invade the brain. In the human body, the GIT mucosal surfaces are the largest and one of the most complex parts of the immune system. The GIT microflora plays an essential role in host health owing to its involvement in nutritional, immunological, and physiological functions [35]. Discrimination between beneficial commensal bacteria, harmless antigens, and pathogenic microorganisms is a fundamental issue in the role that GIT immune cells play in maintaining the balance between immune response and tolerance [36]. Microbial imbalances have been associated with enhanced risk of specific diseases [35]. Under the conditions of disturbed microflora homeostasis, impaired mucosal permeability, and immunocompromisation, microbial translocation is pathologically increased, and then causes systemic inflammatory responses which play an important role in the eventual outcome towards multiple-organ involvement including the brain [37,38].

Peripheral (GIT) infection associated with MS

The microorganism itself can initiate an immune response in GIT. Molecular mimicry has been proposed as an explanation for autoimmune side effects/disorders of microorganism infections including MS [39]. In this respect, current *Hp*-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 possibly perpetuating neural tissue damage in neurodegenerative disorders including MS [3,5,40,41]. Infectious stimuli may also participate in the development of autoimmunity by inducing an increased expression of heat shock proteins, chaperones, and transplantation antigens, which results in abnormal processing and presentation of self antigens; superantigens appear to be one of the most effective bacterial

components to induce inflammatory reactions and to take part in the development and course of autoimmune mechanisms; defective immune system is associated with a higher risk of a development of autoimmune disease [42].

Regarding MS aetiology, an autoimmune mechanism is also suspected based on significant analogies with experimental allergic encephalomyelitis (EAE). In order to induce EAE in animals, both an immunogen and an adjuvant must be injected at the same time. By using immunogens, it is suggested that a microorganism responsible for autoimmune activity in MS could be a normally occurring gut bacterium [39]. Moreover, adjuvant molecules are normally absent in human body fluids or tissue, except the gut [43,44]. Apparently, the two groups of substances, potential immunogens (mimics) and adjuvant molecules, known to be required for an autoimmune response are normally found in the human gut. The cell wall material of the microorganism responsible for a secondary infection is a source of the adjuvant. Although the adjuvant itself cannot cross the gut, the adjuvant–immunogen complex can probably cross the gut barrier, resulting in EAE development. Besides, many potential encephalitogens occur within bacteria and viral cells; the most probable source of these mimics is the normal gut. In addition, bacteria and viruses are powerful inflammatory cytokine stimulators and activators of the complement pathway. They affect vascular permeability; generate nitric oxide (a rapidly diffusing gas and a potent neurotoxin that may contribute to the apoptotic neuronal cell death in degenerative neuropathies); induce proteoglycan synthesis and apoptosis; and possess biological activities [45], which can induce the cascade of events leading to the pathological and biological hallmarks of neuroinflammation.

Incoming bacterial signals include secreted chemoattractants, flagellin, bacterial nucleic acids, and cellular constituents such as lipopolysaccharide (LPS) and peptidoglycans. Specifically, the bacterial inflammatory surface molecule LPS, a bacterial endotoxin, is a powerful inflammatory factor of Gram negative bacteria. LPS and peptidoglycan are highly resistant to degradation by mammalian enzymes and thus may provide a persisting inflammatory stimulus [46]. 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 Guillain-Barré syndrome (GBS) possibly associated with MS [41,47]; chemical analyses of the core oligosaccharides of neuropathy-associated *C. jejuni* strains have revealed structural homology with human gangliosides [48].

The monocytes in GIT express pattern-recognition receptors in the form of Toll-like receptors (TLRs). TLRs are a family of conserved pattern-recognition receptors (PRRs) that recognise pathogen-related molecular patterns and serve as primary sensors of the innate immune system. Ten members of the TLR family have so far been identified in the human genome. The ligands for these receptors are structurally highly conserved microbial molecules including LPS (recognised by TLR4), flagellin (TLR5), lipopeptides (TLR2 in combination with TLR1 or TLR6), CpG motif-containing DNA (TLR9), single stranded RNA (TLR7 and TLR8), double-stranded RNA (TLR3), and profilin present on uropathogenic bacteria (TLR11) [49]. Microbial LPS binds to TLRs and activate innate and inflammatory responses [50]; LPS stimulates cells via the TLR4, causing inflammatory cytokines release and costimulatory molecules upregulation on antigen-presenting cells. The combination of signals from antigens, costimulation, and cytokines allows CD4 T cells to overcome suppressive barriers and accumulate in large numbers. T cells that are primed in an LPS-stimulated environment are programmed for long-term survival following clonal expansion. LPS is recognised for generating Th1 responses. Nevertheless, under appropriate conditions, it can also support differentiation into other T-helper lineages, signifying its pleiotropic nature [51]. In this respect, it has been shown that TLR4 which recognises LPS, downregulates disease severity in EAE and Th17 cell responses, but promotes Th1 cell responses, which may inhibit the differentiation of Th17 cells [51]. Of note, EAE is a Th17-mediated autoimmune disease [51]. Relative recent data indicate that the gut flora may influence the development of EAE in a way that is dependent on Valpha14 invariant NKT cells necessary for maintaining the mesenteric Th17 T cells, thereby playing an important role in the prevention and treatment of autoimmune diseases [52].

In view of the aforementioned data, it appears that GIT microbial infections play a pivotal and diverse role leading to MS pathologies.

Gastrointestinal tract: the "little brain"

The GIT is controlled by the independent enteric nervous system (ENS). It is also strictly connected to the CNS, and a bi-directional communication exists between them. The communication involves neural pathways as well as immune and endocrine mechanisms. The brain–gut axis plays an important role in the modulation of GI functions. Signals from diverse sources (e.g., pain, sound, sight, smell, somatic, and visceral sensations) access the brain. These inputs are modified by memory, cognition and affective mechanisms and are integrated within the neural circuits of the CNS, spinal cord, autonomic and ENS. These inputs can have physiological effects, such as changes in motility, secretion, immune function, and blood flow to the GIT [53].

Specifically, GIT constitutes one of the largest organs whose motor, transport, secretory, storage, and sensory functions are controlled by the nervous system. The two most essential goals of GIT motor and sensory function are the efficient absorption of nutrients and the maintenance of orderly aboral movement of chyme and indigestible residues along the gut. Its motility is also crucially important in preventing bacterial overgrowth in intestine [54]. To obtain these goals, the gut is supplied by intrinsic sensory neurones of the enteric nerve plexuses, forming the ENS, as well as extrinsic spinal and vagal afferent neurones, in close contact with two important non-neural surveillance systems in the mucosa: endocrine and immune cells. With these connections and their sensory modalities, GI sensory neurones are able to recognise subtle changes in the chemical and physical environment within the lumen, interstitial space, vasculature, and muscle of the gut and transform the information into action potential codes [55,56].

Neurones supplying the GIT are designated as afferent or efferent depending on the direction in which they conduct information; information is conducted centrally by afferent neurones and peripherally by efferent neurones [54]. ENS sends numerous afferent projections to CNS and receives from there efferent fibres, reaching the GIT through the extramural sympathetic and parasympathetic nerves synapsing on the ENS neurones, and thus forming the neural brain–gut axis [55–57]. Spinal afferents subserve an emergency function because, in case of an alarm by influxing acid, they stimulate mechanisms of mucosal protection via an efferent-like release of transmitters. Other sensory neurones signal chemical noxae to the brain, a task not confined to spinal afferents because vagal afferents communicate gastric acid and peripheral immune challenges to the brainstem and in this way elicit autonomic, endocrine, affective, and behavioural reactions. Sensitisation may be brought about by inflammatory processes that lead to upregulation and functional alterations of receptors and ion channels on sensory neurones [58]. The external autonomic nerves, such as vagal nerves, contain about 10% of efferent fibres that function mainly as pre-ganglionic cholinergic neurones synapsing with the neurones of ENS. Postganglionic neurones of ENS utilise a variety of neurotransmitters, such as gastrin releasing peptide (GRP), vasoactive intestinal polypeptide (VIP), pituitary adenylate

cyclase-activating peptide (PACAP), and substance P (SP). These neurotransmitters may, for example, act directly on the parietal cell as is the case for acetylcholine (Ach), or they regulate acid secretion indirectly by affecting the release of gastrin from G-cells, somatostatin (SST) from D-cells, histamine from enterochromaffin-like (ECL) cells, ghrelin (Gr) from Gr cells, atrial natriuretic peptide from enterochrochromaffin cells (EC), serotonin from enterochromaffin (EC) cells, and melatonin from entero-endocrine (EE) cells. The majority (over 90%) of vagal fibres are, however, sensory afferents that are either intrinsic (confined to the gastric wall) or extrinsic, both containing and releasing at their terminals neurotransmitters [SP, calcitonin gene-related peptide (CGRP), VIP, PACAP]. There is a good body of evidence that luminal acid or *Hp* adhering to the surface of epithelial cells activate the sensory nerves containing neuropeptides such as GRP, SP, PACAP, or CGRP, which, in turn, activate higher autonomic centres or stimulate locally the GIT mucosa to secrete SST resulting in the inhibition of gastrin secretion and subsequently gastric acid secretion [57,59].

The ENS has been recognised to display many similarities with the CNS [60]. Like the CNS ("big brain"), the ENS is a self-regulating system (often called the "little brain"); it is the largest and most complicated division of the peripheral nervous system [61] and contains over 100 million neurones (motor, sensory, and interneurones) and a widespread cell population of enteric glial cells (EGCs). These cells represent the morphological and functional equivalent of CNS astrocytes within the ENS; EGCs display morphologic and molecular similarities to CNS astrocytes and express neurotransmitter receptors, indicating that, like astrocytes, they are active participants in neuronal communication [62]. EGCs have trophical, protective and modulatory functions toward enteric neurones. Moreover, EGCs seem to be active elements of the ENS during intestinal inflammatory and immune responses, sharing with astrocytes the ability to act as antigen-presenting cells and interacting with the mucosal immune system via the expression of cytokines and cytokine receptors. EGCs may also share with astrocytes the ability to regulate tissue integrity, thereby postulating that similar interactions to those observed for the BBB may also be partly responsible for regulating mucosal and vascular permeability in the GIT [63]. Recently, some evidence indicates that EGCs may be involved in the pathophysiology of enteric neurogliopathies [64]. In this respect, EGCs dysfunction/loss are sufficient to induce enterocolitis and early Crohn's disease associated with alterations of peripheral and/or CNS including syndromes such as MS [65,66]. Moreover, GIT infections, such as typhoid fever, are complicated with unusual neurological disorders including encephalopathy and the mentioned GBS associated with MS [41,47,64–69]; the predilection of typhoid toxins to nervous system is well known [68], and autonomic neuropathy can affect parasympathetic, sympathetic, and enteric nerves or neurones and is an important cause of morbidity and mortality in GBS [70].

From the "little brain" to the "big brain" infection

Afferent neurones linking gut to brain are a site of integration of an impressive collection of endocrine, paracrine, neuronal, and humoral signals. The best recognised pathway for transmission of information from the peripheral immune system to the neuroendocrine system is the humoral in the form of cytokines, although neural transmission through the afferent vagus is also well documented [71]; the peripheral immune message from GIT is transmitted to the brain via a slow humoral pathway and a fast neural pathway resulting in the expression of pro-inflammatory cytokines in macrophage-like cells and microglia in the brain. The humoral pathway involves the production of IL-1 by phagocytic cells in the circumventricular organs and choroid plexus, followed by its diffusion to the brain [72].

The vagal anti-inflammatory reflex

The vagus nerve provides a main neural pathway, and cytokine-responsive vagal afferent neurones participate in the communication between the peripheral immune system and the brain [73]. This is supported by a particular proximity of vagal afferent nerve fibres to immunologically relevant structures such as macrophage-like cells, paraganglia, and connective tissue containing macrophages and dendritic cells [32]. Bacterial LPS (endotoxin) is able to cause release of IL-1β from these cells, subsequently leading to excitation of vagal afferents. Through these properties, vagal afferents are thought to mediate a vago-vagal anti-inflammatory reflex. Peripheral immune and inflammatory signals trigger an input to the brain both via vagal afferents and circumventricular organs that are devoid of a BBB. These signals are processed by the brainstem and central autonomic circuitries to provide an output via cholinergic vagal efferents. Ach, released from efferent axons in the periphery, activates α7 subunit-containing nicotinic receptors on tissue macrophages and other immune cells which results in inhibition of pro-inflammatory cytokine release and suppression of inflammation [74]. It is important to note that autonomic dysfunction is encountered in MS and GBS [75]. While vagus nerve stimulation improves dysphagia and cerebellar tremor in MS [76], impairment of the sympathovagal balance correlates with fatigue, a common symptom in MS patients [77].

Humoral pathway – BBB breakdown

A slow pathway of transmission from the immune system to the brain is represented by the production of molecular intermediates at the level of the blood–brain interface in response to circulating cytokines or microorganism fragments. In response to local infection, the peripheral tissue macrophage induces an inflammatory response through the release of IL-1β and TNF-α. These cytokines stimulate macrophages and endothelial cells to express chemokines and adhesion molecules that attract leucocytes into the peripheral site of infection. Perivascular macrophages are the most reactive cell type and produce IL-1β and TNF-α and play important roles for the initiation and spread of neuroinflammatory processes. The main cellular target for IL-1β and TNF-α produced in the brain (peri)vascular compartment is the endothelium, where these cytokines induce the expression of adhesion molecules and promote leucocyte infiltration [32]. Although the source of infection is exogenous for GIT, it is blood-borne for the CNS, which makes the brain (peri)vascular compartments more prone to infection by pathogens than peripheral organs.

Specifically, regarding the MS association with *Hp*-I, the bacterium may contribute to BBB disruption through induction of inflammatory mediators [e.g., cytokines (IL-1β and TNF-α) and chemokines (IL-8)] [25,29]. Once the BBB breaks, massive infiltration of T cells, increased expression of adhesion molecules on endothelial cell surface, and leakage of inflammatory cytokines and antibodies will aggravate the MS lesions [78]; lymphocyte recruitment into the brain across the BBB vascular endothelial cells represents a critical event in MS pathogenesis, and transendothelial migration of activated leucocytes are among the earliest cerebrovascular abnormalities seen in MS brains and parallel the release of inflammatory cytokines/chemokines [79,80]. The number and intensity of staining of CD163-positive perivascular macrophages and infiltrating monocytes are increased in brains of patients with MS and of animals with EAE [17]. Resident CNS perivascular macrophages display increased expression of adhesion molecules and chemokines for mononuclear phagocytes during EAE [81]. Moreover, chemokine receptors can be found in blood-borne macrophages in the early demyelinating stages of animal EAE and human MS [82]. These observations indicate that resident

perivascular macrophages play an important role in attracting circulating mononuclear cells. In accordance with this idea, it has been shown that depletion of blood-borne macrophages reduces the number of CD163-positive brain perivascular macrophages and attenuates demyelination and the clinical symptoms of EAE [83]. Thus, both infiltrating monocytes and resident perivascular macrophages are involved in the development of the clinical signs in this animal model of MS. Moreover, dendritic cell migration throughout perivascular brain compartments plays an important role in immune invasion of the CNS of T cells; they are the primary antigen-presenting cells directing T-cell functions and are, therefore, particularly important in directing the immune pathology, characteristic of MS [84]. The presence of the aforementioned autoreactive immune effectors, together with activated CNS astrocytes and microglia, lead to demyelination, axonal, and neuronal damage MS [85].

Fast neural pathway

The human CNS is targeted by different pathogens that, apart from trafficking into the brain through infected blood cells, may use a distinct pathway to bypass the BBB by using retrograde axonal transport through sensory or motor fibres. In this regard, the role of the vagus nerves in the transmission of information from the periphery to the brain has been assessed by vagotomy experiments in which the vagus nerves are sectioned under the diaphragm. Using this approach, vagal afferents have been shown to mediate at least partially the neural activation of the brainstem, hypothalamus, and limbic structures in response to peripherally administered LPS and IL-1 [72]. Retrograde axonal transport in the vagus nerve is a fast pathway from GIT to the brain as has been demonstrated for the prion protein PrPSc and several neuropeptides. Prions are orally transmissible agents that induce a devastating subacute neurodegeneration (spongiform encephalopathies, human Creutzfeldt–Jakob disease) when they successfully reach the CNS; in the case of peripheral transmission, such as human consumption of contaminated tissue, the infectious agent uses the sympathetic noradrenergic neurones to reach the CNS after early replication in lymphoid tissues [86]. The disease-specific isoform of the PrPSc is found in the enteric nervous system of the submucosal and myenteric plexus and the gut-associated lymphoid tissue following oral scrapie ingestion; PrPSc is highly resistant to detergent extraction [87]. Prion spread from these sites to the CNS can occur by axonal transport within the parasympathetic nervous system (e.g., from the vagus nerve to the dorsal motor nucleus of the vagus) and the sympathetic nervous system (e.g., from the splanchnic nerve to the intermediolateral cell column of the spinal cord [88,89]; retrograde axonal transport appears to be the faster route of brain invasion [89]. In this respect, *Hp*-I might promote uptake and propagation of alimentary prions from the GIT by upregulation of gastric PrPc expression. This is linked to *Hp*-induced hypergastrinaemia, enhanced IL-1β mucosal production and augmented mucosal prostaglandin synthesis; *Hp* creates a milieu for increased prions' propagation in the GIT [90].

Moreover, axonal transport of several other neuropeptides in the vagus nerve has been demonstrated, e.g., SST, SP, cholecystokinin octapeptide (CCK-8) and VIP [91,92]. The evidence suggests that these peptides are localised to afferent fibres. It seems probable that the synthesised neuropeptides are transported toward the CNS and influence brain function. All aforementioned neuropeptides have been involved in the pathophysiology of GIT infections induced by *Hp* or other pathogens [93–99]. For instance, *Hp*-infected mice exhibit high density of SP and VIP-immunoreactivity nerves in the stomach and of SP in the spinal cord [93], there is a close relationship between *Hp* and gastric SST regulation, and, moreover, SST and/or octreotide by exerting many inhibitory effects on the GIT may promote bacterial translocation [94,96]; VIP appears to display GIT antimicrobial activity [97]; SP plays a role in diarrhoea mediation in HIV patients with naturally occurring chronic cryptosporidiosis [98]; and CCK stimulates intestinal transit time and may prevent the GIT bacterial overgrowth and translocation [99]. Equally, these neuropeptides also appear to be involved in the pathophysiology of MS [100–104]. For instance, grade 3 acute EAE, disrupts the rat striatal neuropeptide SST receptor–effector system, thereby providing new insight into the molecular basis of EAE which may contribute to a better understanding of MS in human [100]; vasoactive neuropeptides (VNs) including VIP, have crucial roles in the CNS as neurotransmitters, vasodilators, immune and nociception modulators, and autoimmunity VNs or VN receptors may affect BBB and Virchow–Robin spaces functions, and, thus, might contribute to the pathophysiology of neurological-related diseases including MS [101]; the occurrence of SP immunoreactive astrocytes in MS suggests that this peptide might be important both in the development of plaques and in governing the natural history of the disease [102]; and augmented amounts of CCK-8 are released together with enhanced aminopeptidase activity in MS [104].

In view of the above-mentioned data, we can conclude that, apart form pathogens' intranasal inoculation and pathogens' trafficking into the brain through infected blood cells, the faster GIT-associated retrograde axonal transport pathway appears to play a crucial role in the pathophysiology of MS. Therefore, future studies are mandatory to elucidate in depth this fast transmission neural pathway that may offer clues for potential therapeutic strategies targeting MS.

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The C-terminal Region of HPNAP Activates Neutrophils and Promotes Their Adhesion to Endothelial Cells

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HPNAP, *Helicobacter*, AGPs, anti-inflammatory therapy.

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Abstract

Entire Helicobacter Pylori Neutrophil Activated Protein (HPNAP) and its truncated forms NH_2-terminal region $HPNAP_{1-57}$ and C-terminal region $HPNAP_{58-144}$ after cloning into pET29c vector, purification and removal of LPS traces were subjected to human neutrophil activation. Our results revealed that the C-terminal region of HPNAP is indispensable for human neutrophil stimulation and their further adhesion to endothelial cells – a step necessary to *H. pylori* inflammation – in a ratio equal to that exhibited by the entire protein.

In addition, experiments concerning the implication of Arabino-Galactan-Proteins (AGPs) derived from Chios Mastic Gum (CMG), the natural resin of the plant *Pistacia lentiscus* var. *Chia* revealed the inhibition of neutrophil activation and therefore their adhesion to endothelial cells, *in vitro*.

Both, the involvement of HPNAP C-terminal region in stimulation-adhesion of neutrophils to endothelial cells as well as the inhibition of this process by AGPs have to be further investigated and may be exploited in a future anti-inflammatory therapy for *H. pylori* patients.

Helicobacter pylori infection is among the most common human infections and the major risk factor for peptic ulcer disease and gastric cancer. The *H. pylori* virulence factors are three conserved antigens, namely the vacillating cytotoxin A (VacA), the cytotoxin-associated antigen (GagA), and *Helicobacter pylori* – neutrophil-activating protein (HPNAP). HPNAP attracts and activates neutrophils, monocytes, and mast cells, resulting in the release of proinflammatory mediators [1]. The same molecule promotes Th1-type immune responses, likely acting as a Toll-like receptor-2 agonist [2]. It has been also reported that the broad C-terminal region of HPNAP stimulates neutrophil activation indicated by the production of reactive oxygen intermediates (ROIs) after nicotinamide adenine dinucleotide phosphate oxidase activation [3].

We present here evidence that the C-terminal region of HPNAP is indispensable for neutrophil adhesion to endothelial cells, a step necessary to *H. pylori* inflammation. In addition we show that arabino galactan proteins (AGPs) derived from Chios mastic gum (CMG), the natural resin of the plant *Pistacia lentiscus* var. *Chia* [4], inhibit neutrophil activation in vitro.

Materials and Methods

Cloning and Purification of the Entire HPNAP and its Truncation Forms

The entire protein HPNAP as well as its truncated forms $HPNAP_{1-57}$ and $HPNAP_{58-144}$ were cloned into pET 29c vector and purified as previously described [3].

Endothelial Cell Preparation and Culture

Endothelial cells were isolated from the umbilical cords of healthy newborns by the collagenase perfusion method as previously described by Jaffe et al. [5] with minor modifications. In brief, the umbilical cord veins were carefully washed with sterile phosphate buffered saline (PBS) (0.9% NaCl, pH 7.4) and were then filled and incubated with Earle 199 medium that contained 0.5 μg/mL collagenase, for 10 minutes at room temperature. The content was then emptied in a clean, sterile falcon and the umbilical cord vein was washed with Earle 199 medium (without collagenase). The total content was then centrifuged for 10 minutes at 800 ***g*** and

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finally resuspended in complete Earle 199 medium (20% fetal calf serum, 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 50 μL Pen/Strep, 0.25 μg/mL fungizon, 50 μg/mL gentamycin, 90 μg/mL heparin). The cells were then placed in flasks precoated with 0.2% w/v gelatin in PBS and cultured at 37 °C until they reach confluence. Before use, the cells were trypsinized with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid, centrifuged for 10 minutes at 800 ***g*** and finally resuspended in complete Earle 199 medium.

Neutrophil Adhesion Assay

Neutrophil attachment was measured on microwells. In particular, 8×10^4 cells in Earle 199 medium (10% fetal calf serum) were placed in each well of polystyrene plates precoated with endothelial cells (EC) (10^6/mL) that had been pre-attached to 1% gelatin. Neutrophils were incubated in the wells for 30 minutes at 37 °C in the presence of 1 μM $HPNAP_{wt}$-6 His, 1 μM $HPNAP_{1–57}$-6 His, and 1 μM $HPNAP_{58–144}$-6 His with or without AGPs isolated from 0.333 g of CMG. After incubation, non adherent cells were discarded by aspiration, and the wells were rinsed three times with sterile PBS (pH 6.0). Neutrophil attachment was quantified using the myeloperoxidase (MPO) assay as previously described [5]. In brief, neutrophils were lysed with 0.5% (w/v) hexadecyltrimethylammonium bromide in PBS (pH 6.0) for 30 minutes at 37 °C, which resulted in the release of MPO from the monocytes. The amount of MPO in the lysate, which reflects the number of neutrophils bound to EC, was measured spectrophotometrically at 450 nm after the addition of 50 μL 0.2 mg/mL dianisidine dihydrochloride in PBS (pH 6.0) containing 0.4 mM H_2O_2 for 15 minutes using an enzyme-linked immunosorbent assay reader.

Lipopolysaccahride Removal

Lipopolysaccharides (LPS) were removed using SiMAG-Polymyxin magnetic beads (Chemicell). The beads (10 mg for each protein) were washed three times with PBS, 0, 05% NaN_3, and the protein solution containing LPS was added to the beads [3]. After a 30-minute continuous mix in 4 °C, LPS was bound on the beads, which were removed using a magnet. Clear protein solutions were transferred to clean tubes.

Results and Discussion

Human neutrophils were separately incubated with HPNAP-6xHis, $HPNAP_{1–57}$-6xHis (N-terminal region), and $HPNAP_{58–144}$-6xHis (C-terminal region) on micro-wells with pre-attached endothelial cells, and their attachment was quantified by using the myeloperoxidase (MPO) assay [5], as described previously. Besides the entire protein and its truncated forms, neutrophils were also incubated through the same manner, with the neutrophil stimulator formylo-Met-Leu-Pro peptide (fMLP) in order to control their "bioactivity." In addition, a synthetic hexa-histidine peptide (6xHis) was also used for neutrophil activation in order to exclude the possibility that the obtained activation was attributed to the existence of the tailed histidines. Figure 1(A) shows that HPNAP-6xHis and the C-terminal region display almost the same ability to promote neutrophil adhesion to endothelial cells, whereas on the contrary, the N-terminal region exhibits a remarkable lack of this ability.

Considering the existence of LPS and their involvement in the activation it is clearly shown that even after their removal the activation effects did not change significantly (Fig. 1A). These results are consistent with previously published data [3], indicating that the 58–144 region of the HPNAP protein is the key component in neutrophil recruitment, activation, and subsequent adhesion to endothelial cells, leading to oxidative burst and inflammation during *H. pylori* infection. Considering recently published data [6] on the safety and immunogenicity of an intramuscular vaccine comprising VacA, CagA and HPNAP, we suggest that the obtained neutrophil activation by the C-terminal region of HPNAP opens new ways for drug design dealing with *H. pylori* inflammation.

Neutrophil Attachment Inhibition by AGPs from CMG

Chios mastic gum and its derivatives were largely used in traditional medicine to ease the discomfort in patients suffering from gastric pain. Its in vitro antibacterial properties against a great variety of bacteria are well established [4,7]. In this study we demonstrate that AGPs extracted from CMG, as described in [4], inhibit the neutrophil attachment to endothelial cells caused by the HPNAP and its C-terminal region. In particular, human neutrophils were incubated with either HPNAP-6xHis or $HPNAP_{58–144}$-6xHis both in the presence and in the absence of AGPs, and their attachment to endothelial cells was investigated as described previously. Figure 1(B) shows the inhibition of neutrophil attachment to endothelial cells after co-incubation of entire HPNAP and its truncated forms (N-terminal and C-terminal) with the AGPs. In particular, bar 3 shows the absence of any influence of AGPs on neutrophil's attachment to endothelial cells. The designation "control" on the figure represents the found attachment of isolated neutrophils to endothelial cells after incubation, without any other addition of proteins or AGPs. The marked percentages of all other combinations

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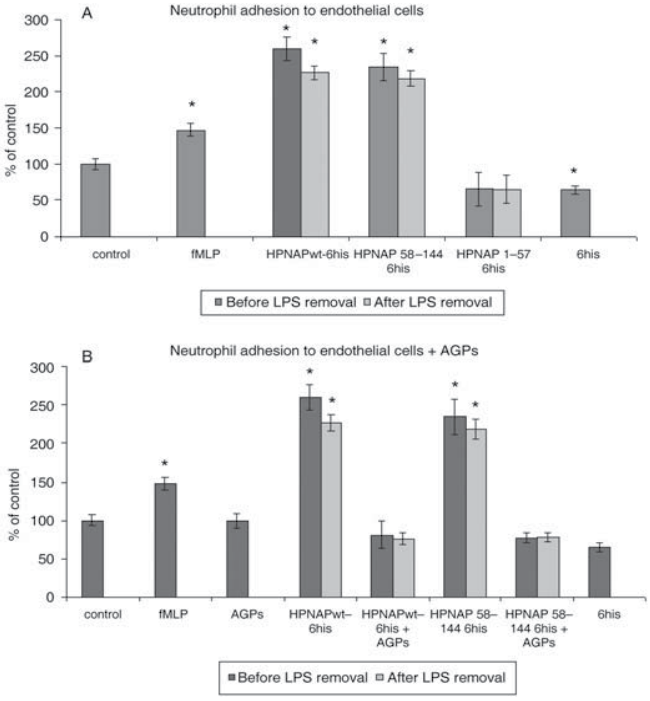

Figure 1 (*A*) Neutrophil adhesion to endothelial cells. Black bars indicate neutrophil adhesion to endothelial cells prior LPS removal while grey bars indicate the adhesion after LPS removal. The data represent triplicates from at least three independent experiments. Error bars indicate standard deviation (SD). Statistical evaluation was performed by Mann–Whitney test. Significant differences with the control values are marked by *. ($p < .001$). **bar 1**, control, **bar 2**, fMLP, positive control of the procedure, **bar 3**, HPNAPwt-6xHis effect on neutrophil adhesion to endothelial cells before and after LPS removal, **bar 4**, $HPNAP_{58–144}$-6xHis effect before and after LPS removal, **bar 5**, $HPNAP_{1–57}$-6xHis effect before and after LPS removal, **bar 6**, 6xHis effect. (*B*) Effect of AGPs on neutrophil adhesion to endothelial cells. **bar 1**, control, **bar 2**, fMLP, positive control of the procedure, **bar 3**, effect of AGPs, **bar 4**, HPNAPwt-6xHis effect before and after LPS removal, **bar 5**, HPNAPwt-6xHis and AGPs coeffect, before and after LPS removal, **bar 6**, $HPNAP_{58–144}$-6xHis effect before and after LPS removal, **bar 7**, $HPNAP_{58–144}$-6xHis and mastic gum extract coeffect, before and after LPS removal, **bar 8**, 6xHis effect.

are calculated by taking into account the control values. Thus, comparison of bars 4 (HPNAP entire) to 5 (HPNAP entire plus AGPs) and 6 ($HPNAP_{58–144}$) to 7 ($HPNAP_{58–144}$ plus AGPs) reveals that neutrophil activation and their subsequent attachment to endothelial cells are inhibited by the AGPs from CMG.

Summarizing our results presented within this work we evidenced that the broad C-terminal region of HPNAP stimulates the neutrophil adhesion and that the AGPs from CMG disrupts the process of neutrophil–endothelial cell attachment caused by HPNAP, an effect that should be further investigated and may be exploited in a future antiinflammatory therapy for *H. pylori* patients.

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Effects of mastic gum *Pistacia lentiscus var. Chia* on innate cellular immune effectors

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Background **The essential oil and Chios mastic gum (CMG) are natural antimicrobial agents currently broadly used in medicine owing to their antimicrobial, antioxidant, and hepatoprotective properties. The aim of this study was to investigate the effect of CMG-extracted arabinogalactan proteins (AGPs/CMG) both *in vitro* and *in vivo*, under the presence of *Helicobacter pylori* neutrophil-activating protein (*HP*-NAP), on the innate cellular immune effectors (neutrophils activations) comparing *H. pylori*-infected patients and healthy controls.**

Patients and methods **The in-vivo effect of AGPs/CMG under the presence of *HP*-NAP in neutrophil activation was investigated in five *H. pylori*-infected patients and three healthy volunteers who received 1 g daily consumption of CMG for 2 months. All participants did not receive any immunosuppressive medication before or during the trial; patients with infectious diseases that could modify their immunologic status were excluded. In-vitro studies with pull-down experiments to assess the effect of AGPs/CMG under the presence of *HP*-NAP on the neutrophil activation were also carried out. Neutrophil activation was estimated by nicotinamide adenine dinucleotide phosphate-oxidase assays and optical microscopy methods by measurement of cytochrome C reduction.**

Results **Neutrophil activation was reduced when incubated *in vitro* with *HP*-NAP ($P=0.0027$) and AGP plus *HP*-NAP ($P=0.0004$) in *H. pylori*-positive patients who consumed AGP for 2 months. Similar results were also obtained when neutrophils were incubated with AGP plus *HP*-NAP ($P=0.0038$) in controls. Pull-down experiments showed a specific binding of AGPs to two membrane proteins of neutrophils, possibly suggesting inhibition of neutrophil activation.**

Conclusion **AGPs/CMG inhibit neutrophil activation in the presence of *HP*-NAP, playing a crucial role in *H. pylori*-associated pathologies in gastric mucosa.**
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Introduction

Mastic is a white, semitransparent, natural resin obtained as trunk exudates from mastic trees. Its scientific name is *Pistacia lentiscus* of the Anacardiaceae family. The plant *Pistacia lentiscus var. Chia* grows particularly and almost exclusively in the southern region of Chios Island, Greece, and produces a resin, known as Chios mastic gum (CMG). CMG has been used in traditional Greek medicine for diverse gastrointestinal disorders including dyspepsia or peptic ulcer disease for more than 2500 years. Ancient Greek physicians (Hippocrates, Dioscorides, Galenos) reported its properties and recommended its use. It is currently used as a seasoning in Mediterranean cuisine, in the production of chewing gum, in perfumery, in dentistry, and by the local population of Chios for the relief of epigastric pain and protection against peptic ulcer disease.

The chemical composition of the mastic oil and the essential oil of the resin have been reported [1,2]; its biological activities can be attributed to a variety of compounds. The essential oil and CMG are natural antimicrobial agents used broadly in medicine in recent years, and current research suggests that they possess beneficial (antimicrobial, antioxidant, and hepatoprotective) properties. In particular, CMG possesses antibacterial activity [1,3,4], and its in-vivo antiplaque action in the oral cavity has been attributed to its inhibitory action against overall bacterial growth [5], especially against *Streptococcus mutants* [6]. Data on the efficacy of *Pistacia lentiscus* against

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Helicobacter pylori and peptic ulcer are controversial. Clinical studies initially indicated that CMG is effective against gastric and duodenal ulcer disease [7,8]; mastic gum proved bactericidal against *H. pylori in vitro*, killing 50% of the strains tested at a concentration of 125 μg/ml, and 90% at a concentration of 500 μg/ml [9,10]. Other studies also reported that a total mastic extract without polymer led to an approximately 30-fold reduction in *H. pylori* colonization of infected mice [11] and the in-vitro inhibition of *H. pylori* has been attributed to isomasticadienolic acid. In contrast, other studies [12,13] showed *H. pylori* resistance after CMG intake in infected mice and humans. Recently, Kottakis *et al.* [14] reported that the in-vitro growth of *H. pylori* was inhibited by adding CMG extracts produced from at least 1.4 g of mastic resin.

Regarding the possible immunomodulatory effect of CMG, it has recently been reported that arabinogalactan proteins (AGPs) from *Echinacea purpurea* stimulate phagocytosis and release tumor necrosis factor by macrophages [15,16], which are innate cellular immune effectors; AGP from *E. purpurea* also possesses complement-stimulating activities *in vitro* [17], and interactions with leukocytes or the complement system have been shown [18,19]. Pharmacological studies on AGPs from various plants showed additional immunomodulatory activities; AGPs enhance the proliferation and IgM production in mouse lymphocytes as well as nitrile and IL-6 production in mouse macrophages [20].

In this study, the effect of CMG/AGPs on innate cellular immune effectors including neutrophils was investigated; the activation of human neutrophils was estimated both *in vitro* and *in vivo* and the relative interaction between *H. pylori* neutrophil-activating protein (*HP*-NAP) and CMG/AGPs was studied through nicotinamide adenine dinucleotide phosphate oxidase assay.

Patients and methods

Patients

This was a 2-part study. Part 1 was designed to evaluate the in-vitro effect of AGPs/CMG on neutrophil activation by pull-down experiments and incubation of AGPs with *HP*-NAP and neutrophils in five *H. pylori*-positive patients (three women, age range 21–74 years) and three *H. pylori*-negative healthy men volunteers (age range 23–72 years).

Part 2 was designed to evaluate the in-vivo effect of mastic gum consumption on neutrophil activation in all participants. The actual gold standard for the diagnosis of *H. pylori* infection was the detection of *H. pylori* organisms on microscopy of mucosal biopsy specimens. Additional routine diagnostic criteria included a positive urea breath test. *H. pylori* detection methods and histopathology were described previously [21].

Each participant signed a consent form before enrollment and the study protocol was approved by the local ethics committee. All participants did not receive any immunosuppressive medications before or during the trial, and they did not suffer from any other infectious diseases that could modify their immunological status. In addition, participants were excluded if they had taken H_2-receptor antagonists, proton pump inhibitors, antibiotics, bismuth compounds, NSAIDs [excluding low doses (80 mg two to three times weekly) of aspirin] in the preceding 4 weeks. Participants were also excluded if they had undergone previous gastric surgery; were on anticoagulant therapy; were alcohol abusers; had gastric cancer or other neoplasms, or had severe cardiac, pulmonary, kidney, or liver disease.

Methods

Participants reported at 09 : 00 h after a 12-h fast. Intravenous sedation was given, and standard upper gastrointestinal endoscopy was performed with a forward viewing videoscope (Olympus, opto-electronics Co., Ltd, CE 0197, Japan) for evaluation of any macroscopic abnormalities. Simultaneously, biopsy specimens were obtained from the antral region within 2 cm of the pyloric ring and from the corpus. The biopsy specimens from each site were placed in 10% formalin and submitted for histological examination. Before endoscopy, venous blood was drawn from each participant and the neutrophils were isolated [22] and incubated with: (a) *HP*-NAP and (b) *HP*-NAP plus mastic gum. All participants were subsequently treated with mastic gum 1 g daily for 2 months and the neutrophils were subjected to the same procedure to evaluate the effects of mastic gum consumption on the modification of in-vitro activation of neutrophils by *HP*-NAP and mastic gum; they were subjected to simultaneous incubation with *HP*-NAP and mastic gum because of the fact that *in vivo* (in gastric mucosa) they are exposed simultaneously to *HP*-NAP and mastic gum activation.

Specifically, in an attempt to investigate the effect of CMG/AGPs on the innate cellular immune effectors including neutrophils, experiments concerning their interaction with neutrophils of the participants were performed; their possible specific interaction with isolated membrane proteins from neutrophils was investigated by pull-down experiments.

The amount of superoxide anions produced by neutrophils was measured through superoxide dismutase (SOD)-inhibitable reduction of cytochrome C at 550 nm.

Six measurements of cytochrome C reduction were obtained from each participant at baseline and at 2-month posttreatment period; three measurements with the presence of *HP*-NAP and three with the presence of

HP-NAP and CMG. Specifically, each variable (i.e. neutrophil activation after incubation with *HP*-NAP and *HP*-NAP/CMG) was measured thrice in each participant at baseline and at posttreatment period with negligible difference between the three measurements; a total of nine and 15 values pertaining to the three controls and five patients, respectively, were compared. The intrasubject variability of the values measured in each control and patient was very low (3–5%) because of the high precision and reliability of the method used, thereby supporting the high reproducibility of the values measured.

Extraction of arabinogalactan proteins

Dry Chios mastic gum (resin) was pulverized to a fine powder, 5 g of which were mixed with 0.1 mol/l NaCl, 20 mmol/l Tris–HCl, pH 7.5 and after overnight stirring at 4°C, the mixture was centrifuged to separate soluble (supernatant) from insoluble (pellet) material as described previously [14]. The soluble material was filtered by using 0.45-μm HA filters and dialyzed extensively against water.

Production of polyclonal antibodies

Antiserum to AGPs was produced using the isolated extracts. A 3-month-old rabbit was injected subcutaneously with the extracts from 1 g of mastic as described previously [23]. The resulted rabbit serum was collected after the second and third injection and was stored in 1 ml aliquots at −20°C.

Electrophoresis and western blotting

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed according to Laemmli [24] in 12% polyacrylamide gels and protein transfer onto immobilon membranes was followed by immunostaining using the produced antiserum in a dilution 1 : 1000.

Isolation of human neutrophils

Human neutrophils were prepared from buffy coats of venous blood of healthy donors as described previously [22]. The procedure is a modification of the method of Boyum [25] and it includes centrifugation of cells in Ficoll medium and sedimentation of the mixture in dextran solution T-500 6% w/v. Erythrocytes remaining in the granulocyte fraction were removed by lysis in a 0.8% w/v solution of NH_4Cl in H_2O. After incubation in NH_4Cl for at least 10 min, the cells were centrifuged at 400 *g*, and the supernatant was discarded. The lysis and centrifugation were repeated until the preparation was free of erythrocytes. This procedure usually results in granulocyte fractions with neutrophil contents of greater than 95%.

Isolation of membrane proteins from human neutrophils

Human neutrophils were suspended in lysis buffer that contained 10 mmol/l Pipes pH 7.0, 10 mmol/l KCl, 3 mmol/l NaCl, 4 mmol/l $MgCl_2$, 5 mmol/l sodium pyrophosphate, 25 mmol/l sodium fluoride, 1 μg/ml pepstatin, and 1 mmol/l PMSF, were sonicated three times (12 s each time) and centrifuged (1000 *g*) to remove nuclei. The supernatant was transferred onto centrifuge tubes filled with sucrose cushions ranging from 15 to −34% w/v in lysis buffer, and was centrifuged at 120 000 *g*, for 45 min at 4°C. The membrane proteins between the sucrose cushions were removed, resuspended in four volumes of lysis buffer and recentrifuged at 200 000 *g* again for 45 min at 4°C [26].

Biotinylation of mastic gum proteins

The procedure for glycoprotein oxidation was described previously [27]. The protein extract (from 1 g of mastic gum) was dissolved in 250 μl of 0.1 mol/l Na-acetate buffer, pH 4.5 and precooled on ice. A fresh stock of 0.5 mol/l $NaIO_4$ was prepared, precooled on ice and stored in the dark. $NaIO_4$ solution was added to the sample to a final concentration of 10 mmol/l periodate and the mixture was incubated in the dark for 1 h at room temperature. The reaction was stopped by adding 1/10 volume of 0.5 mol/l ethylene glycol and the excess of the glycol reagent was removed by dialysis against PBS.

The biotinylation of oxidized glycoproteins with the commercially available biocytin hydrazide (Pierce Chemical, Rockford, Illinois, USA) was performed as described [28]. Freshly prepared stock solution of biocytin hydrazide (1 mg/ml in dimethyl sulfoxide) was added to the dialyzed sample at a ratio 1 : 10. This was followed by incubation of the samples at room temperature for 1 h, and the resulting biotinylated glycoproteins were analyzed on 12% SDS-PAGE, were transferred on a nitrocellulose membrane, and the biotin was detected with the streptavidin-horseraddish peroxidase protein.

Binding experiments of neutrophil membrane proteins and arabinogalactan proteins

The aforementioned labelled AGPs with biotin were incubated with streptavidin-biotin beads and the membrane neutrophil proteins for 1 h at 4°C. Of note, neutrophil membrane proteins first passed through streptavidin-sepharose beads to exclude any nonspecific binding, and after a brief vortex, the supernatant was used for the binding experiments. After the incubation the centrifuged beads were analyzed onto SDS–PAGE 12% and the protein bands were stained with silver staining.

Neutrophil nicotinamide adenine dinucleotide phosphate-oxidase activity

The amount of superoxide anions produced by neutrophils was measured through the SOD-inhibitable reduction of cytochrome C as described previously [29]. In brief, neutrophils (10^6) were incubated with 1 mg/ml cytochrome C in the presence of 1 μmol/l *HP*-NAP or extract of 1/3 g of mastic gum with or without 20 μg/ml SOD at 37°C for 30 min after rapid centrifugation. The optical density of the

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supernatant was determined spectrophotometrically at 550 nm. The amount of superoxide anions was measured as the difference in optical density of those incubated with or without SOD and each sample was assayed in triplicate.

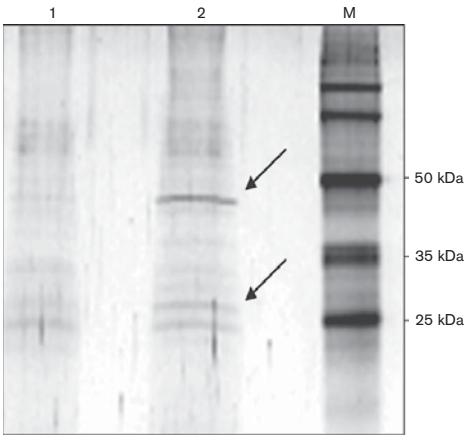
Statistical analysis

The Mann–Whitney *U* test was applied to compare the neutrophil activation as indicated by measurement of cytochrome C reduction between baseline and 2 months posttreatment period. Significance was set at *P* value of less than 0.05.

Results

The specific binding of CMG/AGPs was investigated with pull-down experiments and optical microscopy. The isolated membrane proteins from human neutrophils were passed through streptavidin-sepharose beads and the flow through was incubated with beads of the same column to which were immobilized biotynilated CMG/AGPs. The bound proteins were analyzed by 12% SDS-PAGE and visualized with silver staining. Two bands with MW 25 and 45 kDa were detected (Fig. 1). Their appearance was reproducible and an artificial comigration or binding was excluded. The amount was, however, not enough to characterize them by protein chemical methods determining the receptor family to which they belonged.

Fig. 1



Pull-down experiments of neutrophil-isolated membrane proteins with Chios mastic gum (CMG)/arabinogalactan proteins (AGPs) immobilized on streptavidin-sepharose beads. Proteins were analyzed on SDS-PAGE 12% and visualized by silver staining. M, protein markers, lane (1), membrane proteins nonspecifically bound to the beads and lane (2), specifically bound membrane proteins to immobilized AGPs onto streptavidin-sepharose beads. The arrows at 25 and 45 kDa show two possible membrane proteins, 'candidates' for specific interaction with CMG/AGPs.

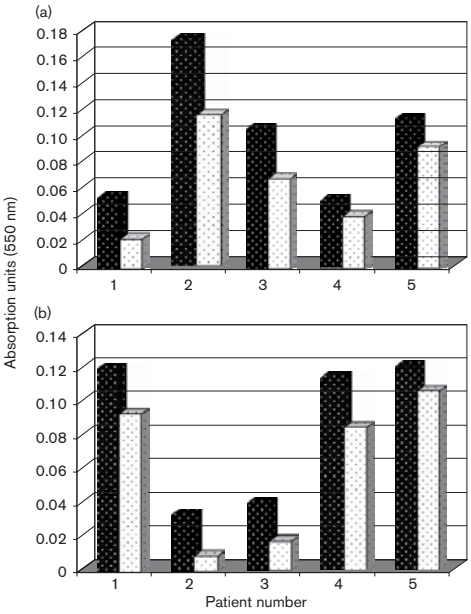
Neutrophil activation was reduced when incubated *in vitro* with *HP*-NAP ($P = 0.0027$) and AGP plus *HP*-NAP ($P = 0.0004$, Table 1, Fig. 2) in *H. pylori*-positive

Table 1 Comparison of neutrophil activation with *HP*-NAP, and *HP*-NAP plus extracted arabinogalactan proteins from AGPs/CMG at baseline and after 2-month CMG treatment in three healthy volunteers (group A) and in five patients with *Helicobacter pylori* infection (group B)

Mean ± SD[a] and significance	Group A ($n=3$)		Group B ($n=5$)	
	Prior	2 Months	Prior	2 Months
HP-NAP	0.065 ± 0.031	0.071 ± 0.029	0.082 ± 0.05	0.066 ± 0.046
	0.0613		0.0027	
HP-NAP/AGP	0.066 ± 0.056	0.026 ± 0.029	0.098 ± 0.044	0.078 ± 0.043
	0.0038		0.0004	

AGPs, arabinogalactan proteins; CMG, Chios mastic gum; *HP*-NAP, *Helicobacter pylori* neutrophil-activating protein.
[a]Six measurements were made (three prior and three after 2-month treatment) in each participant; a total of nine and 15 measured values of neutrophil activation before and after AGPs/CMG treatment were compared between healthy controls and patients, respectively.

Fig. 2



Neutrophil activation of patients after consumption of Chios mastic gum (CMG) for 2 months. The numbers 1–5 indicate the patients enrolled. (a) Neutrophil activation of patients after incubation with *Helicobacter pylori* neutrophil-activating protein (*HP*-NAP) and arabinogalactan proteins. Black and white bars show the activation of neutrophils before and after CMG intake, respectively. (b) Neutrophil activation of patients after incubation with *HP*-NAP. Black and white bars show the activation of neutrophils before and after CMG intake, respectively.

patients who consumed AGP for 2 months. Similar results were also obtained when neutrophils were incubated with AGP plus *HP*-NAP ($P = 0.0038$) but not with *HP*-NAP ($P > 0.05$) in controls.

The only 72-year-old male patient who received 80 mg aspirin two to three times weekly exhibited a similar profile with the other four patients in all the variables measured (no. 5 patient, Fig. 2).

Discussion

To our knowledge, this study shows for the first time that CMG inhibits *HP*-NAP-induced neutrophil activation involved in the pathogenesis of *H. pylori*-related gastric pathologies including peptic ulcer disease and malignancy.

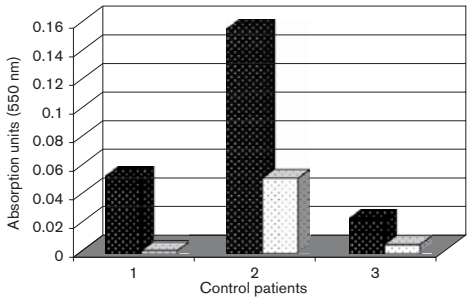
Importantly, *H. pylori* virulence factors promote the release of various chemoattractants/inflammatory mediators including mainly the neutrophil attractant chemokine IL-8 and *HP*-NAP (150 kDa) [30]; an *H. pylori* candidate activating the neutrophils by a similar manner like CMG/AGPs is *HP*-NAP [31]. Specifically, *H. pylori* seems to achieve its pathogenetic role by triggering an intense leukocyte infiltration of the gastric mucosa, and neutrophil activation provides a major source of reactive oxygen metabolites, which can cause tissue damage mainly in the absence of antioxidants [30]. Circulating leukocytes are recruited to sites of inflammation by a well regulated and coordinated process that largely occurs in postcapillary venules. Adhesion molecules are expressed on the surface of endothelial cells, and leukocytes serve to ensure an orderly sequence of cell-to-cell interactions that sustain leukocyte adherence to vascular endothelium and the subsequent transendothelial migration into inflamed tissue. Transcriptional factors [i.e. nuclear factor (NF)-κB] are involved in the expression of endothelial adhesion molecules and in inflammation and carcinogenesis; longstanding *H. pylori*-associated gastritis predisposes to gastric cancer development and reactive oxygen metabolites play a part in *H. pylori*-related gastric carcinogenesis [30]. Thus, CMG might alter the pathophysiology of *H. pylori*-induced upper gastrointestinal tract (GI) pathologies by inhibiting neutrophil activation.

It has been shown that *HP*-NAP binding to neutrophils is inhibited by sialic acid–sphingolipid derivatives [31–33]. Concerning the coexistence of *HP*-NAP and CMG/AGPs in an experimental vial in this study (Fig. 2), the decrease in neutrophil activation could point out a function mechanism like that obtained for sphingolipid–sialic acid derivatives. Namely, in the presence of the inhibitor the antigenic protein *HP*-NAP binds to it (AGPs) and not to neutrophils leading to a decreased activation and a similar mechanism takes place in the gastric mucus. A possible 'in-vivo' mechanism could explain a probable implication of the lymphocytes that, by changing the neutrophil outer membrane receptors after the consumption of AGPs, lead to neutrophil decreased activation [14]. It has to be addressed that the activation took place *in vitro* by using the isolated activators before and after the consumption. All other constituents were the same (recombinant *HP*-NAP and CMG/AGPs) and therefore no other factors except for a change in the cell targets could be different. Apart from sialic acid–sphingolipid derivatives, other relative agents or substances that inhibit *HP*-NAP binding to neutrophils or other *H. pylori*-related neutrophil attractants remain to be defined; these agents might potentially be introduced as additional therapeutic regimens against *H. pylori*-associated pathologies.

Extending this consideration, recent evidence also indicates that CMG contains compounds, which inhibit proliferation and induce death of HCT116 human colon cancer cells *in vitro*. CMG treatment induces cell arrest at G 1 phase, detachment of the cells from the substrate, activation of procaspases 8, 9, and 3, and several morphological changes typical for apoptosis in cell organelles [34]. In this respect, apart from the upper GI tract, *H. pylori* infection may also be involved in the pathogenesis of colon oncogenesis [35,36]. Therefore, these findings suggest that CMG might play a role as a chemotherapeutic agent for the treatment of human gastric, colon, and other cancers [34,37]. For example, CMG inhibits the proliferation and blocks the cell cycle progression in prostate cancer PC-3 cells by suppressing NF-κB activity and the NF-κB signal pathway [37]. More in-vitro and in-vivo studies of the potential anticancer activities of CMG are, however, warranted.

Further supporting the aforementioned considerations, an additional interesting finding of this study shows that the

Fig. 3



Neutrophil activation after their incubation with *Helicobacter pylori* neutrophil-activating protein and arabinogalactan proteins before and after Chios mastic gum (CMG) consumption for 2 months. Black and white bars show the activation of neutrophils before and after CMG intake, respectively. The numbers 1–3 indicate the three control participants.

in-vivo consumption of CMG induces a significant reduction in neutrophil activation when incubated with AGP plus *HP*-NAP in *H. pylori*-infected patients and controls (Fig. 2 and 3, respectively); CMG also induces a significant reduction in neutrophil activation when incubated with *HP*-NAP in *H. pylori*-infected patients. Early studies involving mastic administration to rats with experimentally induced gastric and duodenal ulcers showed a considerable decrease in free acidity [8]. In addition, a double-blind clinical trial carried out in patients with symptomatic and endoscopically proven duodenal ulcer also showed increased symptomatic relief in patients on mastic (1 g daily) compared with patients on placebo, with endoscopically proven ulcer healing in 70% of the patients on mastic [7]. Moreover, mastic gum has been reported to possess considerable in-vitro antibacterial and antifungal activities; it was specifically reported to be effective against *H. pylori in vitro* [10]. In a recent in-vivo study of *H. pylori* infection, however, the activity of mastic gum was compared with antibiotic eradication regimens, and after a 7-day therapy no eradication of the bacterium from the stomachs of mice, which received mastic was noticed [13]. *H. pylori*-positive patients treated with mastic capsules for 7 days, also remained *H. pylori* positive at posttreatment period, suggesting that no 'antibiotic-like' activity should be expected from crude mastic [12]. In contrast, the current study showed that the administration of total mastic extract without polymer seems to be effective in reducing *H. pylori* colonization and that the major triterpenic acids in the acid extract may be responsible for such an activity [11]. These contradictory results could be explained by the different populations studied, methodologies and/or *H. pylori* strains. Therefore, further relative large-scale studies are needed to substantiate the potential benefit of CMG consumption in upper and lower GI *H. pylori*-associated pathologies.

Regarding the possible mechanism(s) of inhibition of neutrophil activation by CMG, pull-down experiments in this study showed, for the first time, a specific binding of AGPs to two membrane proteins of neutrophils, possibly resulting in inhibition of neutrophil activation. Although these two neutophil proteins were not characterized in this study, further studies are needed to elucidate their characteristics and involvement in neutrophil activities.

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Editorial

Helicobacter pylori: an intruder involved in conspiring glaucomatous neuropathy

Jannis Kountouras

Currently, glaucoma is recognised as an optic neuropathy and defined as ''ocular'' Alzheimer disease (AD); it is a chronic neurodegeneration of the optic nerve, the second cause, after cataract, of world blindness and of major public health importance.[1–3] Selective death of retinal ganglion cells (RGC) is the hallmark of glaucoma, also associated with structural changes in the optic nerve head; death of RGC after axonal injury can be induced by a variety of different stimuli.[4] The process of RGC death is thought to be biphasic: a primary injury responsible for initiation of damage followed by a slower secondary degeneration related to noxious environment surrounding the degenerating cells. A working knowledge of the environmental risk factors for the induction and progression of the disease is essential to our clinical practices and helps those patients at greater risk of disease progression and blindness; a major priority is to achieve a better understanding of the risk factors, likely to involve gene–environment interactions.

Evidence for the possibility of an infectious agent comes from reports in younger patients with exfoliative glaucoma (XFG) after intraocular surgery or trauma with iris surgery in infancy and childhood or after penetrating keratoplasty from elderly donors.[5] Moreover, in the middle Norway eye-screening study, the prevalence of XFG in both members of married couples is significantly higher than expected,[5] thereby suggesting a common environmental (probably infectious) agent, which may be of aetiological significance for the XFG development.[6] In this respect, Koch's postulates regarding a causal association with a disease seem to apply in glaucoma,[7] but until recently, no one had associated any micro-organisms with glaucoma. Specifically, although degenerative diseases, including glaucoma or AD, have an increasingly high impact on aged population, their association with *Helicobacter pylori* infection (*Hp*-I) has not been thoroughly researched. Current *Hp*-I appears to induce irregular humoural 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 the apoptotic neural tissue damage observed in neurodegenerative diseases including glaucoma.

In 2001, by using histology, representing the practical gold standard for *Hp*-I diagnosis, we documented for the first time a high prevalence of *Hp*-I in Greek patients with primary open-angle glaucoma (POAG) and XFG, establishing a relationship between *Hp*-I and glaucoma.[8] These results may indicate either a common factor that causes susceptibilities to both glaucoma and *Hp*-I or that *Hp* may be a causal factor for developing glaucoma. In a subsequent study,[9] we reported a beneficial effect of *Hp* eradication on glaucoma progression, suggesting a possible causal link between the bacterium and glaucoma. Moreover, we reported an increased *Hp*-specific IgG antibody concentration in the aqueous humour of patients with POAG and XFG; the concentration of this antibody correlated with the degree of vertical cupping, possibly indicating the severity of glaucomatous damage.[10] We also obtained comparable data for AD which is associated with and shares similar risk factors and pathogenetic mechanisms with glaucoma.[3 4 11–14] Indeed, an association between *Hp*-I and AD/mild cognitive impairment patients in a Greek cohort has been found, *Hp* eradication may positively influence AD manifestations, and *Hp*-specific IgG antibody levels are increased in the cerebrospinal fluid (CSF) of AD patients; its concentration in CSF might reflect the AD severity, thereby supporting a role for this common infection in the pathobiology of the disease.[11–14]

In this issue, Izzotti and colleagues ***(see page 1420)*** collected data in order to elucidate any ethnic correlation between glaucoma and *Hp*-I and whether both diseases share common pathogenetic mechanisms, mainly oxidative injury.[15] Consistent associations were found between Greek data and POAG and/or XFG data from China (using the ^{13}C-urea breath test), Turkey (using serology) and Iran (investigating aqueous humour antibody concentrations). Regarding the data from China, it is important to note that, because *Hp* prevalence is significantly lower in gastro-oesophageal reflux disease (GORD) patients from East Asia, including China, than in those from Western countries, possibly indicating a protective role against GORD, comparable data did not explain its association with glaucoma development in the Chinese population.[16] Additional recent data from India also indicate significantly higher *Hp*-specific IgG antibody levels in POAG patients, supporting the hypothesis on the role of these antibodies in causative mechanisms for glaucoma.[17] In contrast, in their current paper, Izzotti and colleagues report that two studies from Canada and Israel found no association between seropositivity for *Hp*-I and the occurrence of glaucoma. However, concerns regarding mainly the selection of control groups in the latter studies might explain, at least partly, such discrepancies.[18 19] For instance, seasonal variations in the prevalence of *Hp*-I (a significant decrease in *Hp* prevalence during the summer compared with winter) observed in Israel[20] should have been taken into consideration in order to determine the prevalence of *Hp*-I in both glaucoma patients and control subjects. Moreover, *Hp* positivity in Israel is associated with ethnic variations (Sephardic (Asian and African origins) vs Ashkenazi (European and American origins)), which determine the prevalence of *Hp*-I in glaucoma patients and control participants.[19] In addition, differences might exist particularly in the prevalence of XFG glaucoma between Greek and other populations, with a high incidence in Greece and Scandinavia.

In view of the aforementioned data, geographical and ethnic similarities or differences in *Hp* prevalence in glaucoma patients appear to exist. Therefore, further relative large studies should be considered, particularly in populations at greater risk of *Hp*-I and glaucoma development and progression,[21 22] before reaching a final conclusion. The last is essential because eradication of *Hp*-I might delay the progression of glaucoma and other glaucoma-associated neurodegenerative diseases,[23–29] particularly at early disease stages.

Izzotti and colleagues described a comprehensive focus on *Hp*-induced oxidative

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Editorial

stress involved in the pathogenesis of neurodegenerative diseases, mainly including glaucoma. Importantly, oxidative stress is an essential underlying cause of neuroinflammatory and neurodegenerative diseases including glaucoma and blood–brain barrier (BBB) damage connected to them; oxidative stress activates protein tyrosine kinase and matrix metalloproteinases resulting in BBB dysfunction.[30 31]

Specifically, a series of factors have been implicated in inducing BBB disruption, including inflammatory mediators (eg, cytokines and chemokines induced by *Hp*-I) and oxidative stress.[32 33] *Hp* could indirectly affect the brain and other target organs, for example the heart, through the release of numerous cytokines such as tumour necrosis factor (TNF)-α acting at a distance; TNF-α is involved in BBB disruption through a mechanism involving matrix metalloproteinases upregulation.[34] TNF-α and interleukin (IL)-6 (TNF-α is the main trigger for the production of IL-6 by a variety of cells) play important roles in the regulation of the synthesis of other acute phase proteins which are established risk factors for atherosclerosis, such as fibrinogen and factor VIII. These cytokines also have profound effects on lipid metabolism directly at the site of the atherosclerotic lesion but could influence the atheroma process through blood circulating levels, distant production of cytokines, or through stimulating circulating white blood cells to produce them, thereby contributing to BBB disruption and pathogenesis of heart and brain neurodegenerative diseases.[35–37] In addition, *Hp*-induced cytotoxin VacA exhibits chemotactic activities to the bone marrow-derived mast cells (BMDMCs) and induces BMDMCs to produce proinflammatory cytokines including TNF-α;[38] BMDMCs reside adjacent to blood and lymphatic channels, mainly under epithelial surfaces including the BBB and gastrointestinal tract.[39] *Hp* stimulates mast cells directly or via gastrin induction, and mast cells are actively involved in the pathogenesis of *Hp*-associated pathologies.[39] Apart from activated mast cells, vascular endothelial growth factor (VEGF), IL-8, chymase or tryptase (a serine endoprotease released by mast cells) and mast cell growth factor linked to *Hp*-I,[32 39 40] 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.[41]

BBB disruption could play an important role in promoting entry of immune cell infiltration and pathogens into the brain resulting in the development of brain pathologies.[42] 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[42] and possibly glaucoma; infectants including *herpes simplex virus 1*, *Chlamydia pneumoniae* and even *Borrelia* species have been found in brain regions demonstrating significant AD pathology. Besides, autoimmune injury to the optic nerve may occur directly by autoantibodies or indirectly via a ''mimicked'' autoimmune response to a sensitising antigen, which, in turn, damages retinal ganglion cells. 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.[10] Specifically, comparable data could also be considered in the presence of blood–ocular barrier dysfunction. In this respect, *Hp* antibodies circulating in the bloodstream can enter the aqueous circulation due to blood–ocular barrier disruption possibly contributing to glaucoma development and progression.[10] Certainly all the aforementioned speculations merit future in-depth investigation.

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Cover illustration

Electric Eyes: Wirtz iontophoresis electrodes

The "shock" experienced by touching certain fish (electric fish) is described in ancient Egyptian texts dating from 2750 BC.[1] Arab physicians attempted to provide relief from pain of gout and headaches by instructing patients to touch these fish in the expectation that the energy thus transferred would cure them of their ailment. The first study of electricity applied to the eye was in 1855 by the Frenchman B Duchenne. He noted that a continuous application of 1.4 milliamps of current to the eye affected light and colour sensitivity. The visual field for both white light and colour appeared to enlarge. Certain responses in the muscles were also noted.

Drug penetration into the eye was a recognised issue even in the early days. The exposed location of the eye prompted attempts to use electricity to improve penetration of medicines, a practice referred to as cataphoresis (iontophoresis). Certain ions like zinc, copper and mercury could penetrate deeper into the eye with the assistance of an electric current. In 1908 Dr Robert Wirtz used iontophoresis for the treatment of certain eye diseases.[2] The photograph on the front cover shows the special set of variously shaped cathodes that Wirtz designed. Because of the extensive area of contact, a large surface was available for the therapeutic agent to penetrate to the internal part of the eye (figure 1). The handles were made of celluloid with the current entering one end while the other was covered by thick layers of muslin saturated with the dissolved medication being used. It was emphasised that the medication should be diluted in distilled water to facilitate flow of electricity. Wirtz was particularly encouraged by the beneficial effect he achieved in serpiginous ulcer of the cornea which he treated with 0.5% zinc sulphate for one minute at 2 milliamps. Interstitial keratitis was treated with 1% sodium iodide solution and for episcleritis he used chlorine ion from a 0.9 % sodium salt solution.

The success of iontophoresis encouraged others to develop alternative designs. One consisting of glass cylinders with different end shapes that could be filled with the desired solution was developed by Stocker and Birkhauser. This method of therapy was short-lived and by 1920 the treatment by iontophoresis had become unfashionable and rarely used. Today, electricity is used for cutting and coagulating tissue and destroying the roots of undesired hair follicles. Study of the eyes' internal electric currents is widely used in electro-diagnostics and has even been put forth as an explanation for the "whorl" or vortex patterns seen on the corneal surface.[3]

Figure 1 The line drawings illustrate the application of the electrodes and the large area of contact.

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INCREASED CEREBROSPINAL FLUID HELICOBACTER PYLORI ANTIBODY IN ALZHEIMER'S DISEASE

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Background: Helicobacter pylori (H. pylori) infection may play a role in Alzheimer's disease (AD). Aim: A prospective, nonrandomized, comparative study was carried out to examine the levels of anti-H. pylori-specific IgG antibodies in the cerebrospinal fluid (CSF) and serum of AD patients, compared with those of age-matched cognitively normal controls. *Patients:* CSF was aspirated from 27 AD patients and 27 age-matched cognitively normal patients with prostate hyperplasia or long-bone fractures necessitating surgery after epidural anesthesia. Serum samples were obtained from AD patients and the day before surgery from controls. *Methods:* CSF and serum anti-H. pylori IgG concentrations were measured by means of an enzyme-linked immunosorbent assay. *Results:* The mean concentration of anti-H. pylori-specific IgG was significantly greater in (a) the CSF of AD patients (10.53 ± 12.54 U/mL) than in controls (8.63 ± 8.01 U/mL, $p = 0.047$), and (b) the serum of AD patients (30.44 ± 33.94 U/mL) than in controls (16.24 ± 5.77 U/mL, $p = 0.041$). CSF anti-H. pylori IgG antibodies correlated with the degree of severity of the disease. *Conclusion:* H. pylori-specific IgG antibody levels are significantly increased in CSF and serum of AD; its titer in CSF might reflect the AD severity, thereby supporting a role for this common infection in the pathobiology of the disease.

Keywords Alzheimer's disease, anti-H. pylori-specific IgG antibodies, cerebrospinal fluid, Helicobacter pylori, molecular mimicry

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, accounting for 50–60% of all cases (Ferri et al., 2005). The dysregulation in the metabolism of amyloid precursor protein and consequent deposition of amyloid-β (Abeta) peptide has been envisaged as crucial for the development of neurodegeneration in AD (Aisen, 2005; Blasko & Grubeck-Loebenstein, 2003; Watson et al., 2005); key features include the deposition of the Abeta in the form of senile (or amyloid) plaques, formation of neurofibrillary tangles, and loss of neurons and synapses in specific brain regions (Buttini et al., 2005).

A variety of risk factors have been implicated in pathogenesis of AD, including aging, presence of apolipoprotein E4 allele, as well as factors associated with vascular disease including hypercholesterolemia, hypertension, and diabetes (Mayeux, 2003). Moreover, cellular immune defective and apoptotic mechanisms play an important role in the neurodegenerative process in AD (Kountouras et al., 2007); the interaction of activated CD4+T cells with microglia led to a proinflammatory T helper type 1 (Th1) response with a Th1-type cytokine expression profile involved in the pathogenesis of AD via apoptosis representing an important contributor to induction, progression, and pathology of neurodegeneration in AD (Kountouras et al., 2007). In this regard, recent evidence suggests that the possible presence of anti-neuronal antibodies and autoimmune mechanisms might be responsible for eliciting neuronal cell death in AD (D'Andrea, 2005).

Helicobacter pylori (H. pylori) has been implicated in numerous extra-digestive conditions, including ischemic heart disease (Singh et al., 2002), cerebrovascular disorders (Grau et al., 2001), and vascular disorders (Gasbarrini et al., 1999) also associated with AD (de la Torre, 2006; Miklossy, 2003). The relationship between H. pylori infection and gastric autoimmunity is now well established, and H. pylori is thought to be associated with the development of autoimmune sequelae observed in neuropathies (Moran & Prendergast, 2001; Parente et al., 2001), and, moreover, with some autoimmune conditions such as Sjögren's syndrome (Aragona et al., 1999; Showji, Nozawa, Sato, & Suzuki, 1996). Recently, a higher seropositivity for anti-H. pylori immunoglobulin (Ig)G antibodies was reported in 30 patients with AD than in age-matched controls (Malaguarnera et al., 2004). However, this serological test has limitations because it does not discriminate between current and old infections

(Fennerty, 1994). Such a distinction is essential because current H. pylori infection induces humoral and cellular immune (predominant H. pylori-specific Th1 response with a Th1-type cytokine production leading to gastric epithelial cell apoptotic damage) responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves (Kountouras et al., 2005), thereby affecting or perpetuating neural tissue damage. Moreover, eradication of H. pylori infection might hypothetically delay AD progression, particularly at early disease stages. Based on the histological analysis of gastric mucosa biopsy for the documentation of current H. pylori infection, we reported a higher rate of infection in AD patients compared to anemic controls (Kountouras et al., 2006). It is thus reasonable to further investigate the role of H. pylori in AD initiation, progression, or susceptibility by documenting its qualitative and quantitative presence in the cerebrospinal fluid (CSF) of these patients. We therefore investigated the presence of H. pylori-specific IgG antibodies in the CSF of patients with AD and compared their levels with those of age-matched controls to determine whether H. pylori plays a role in this disease.

MATERIALS AND METHODOLOGY

Participants

We prospectively investigated the presence of anti-H. pylori-specific IgG antibodies in CSF and serum samples obtained from 27 AD patients (Group A). The control group consisted of 27 consecutive, age-matched, cognitively normal patients with prostate hyperplasia or long-bone fractures necessitating surgery after epidural anesthesia (Group B). All participants were enrolled consecutively and were native Greek citizens living in Thessaloniki. They were of similar education and socioeconomic status, matched age and sex, and did not belong to high-risk professional groups such as nurses or physicians.

Patients fulfilled the National Institute of Neurological Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA) criteria for the diagnosis of AD (McKhann et al., 1984). All participants underwent a comprehensive neuropsychological examinations including mini-mental state examination (MMSE), Cambridge cognitive test (CAMCOG), functional rating scale of the severity of dementia (FRSSD), neuropsychological inventory (NPI), and geriatric depression scale (GDS). Inclusion of cognitively normal participants required MMSE score >24, CAMCOG score >85, and FRSSD score <5. Moreover, in addition to scores above cutoff value in cognitive tests, subjective memory complaints should be absent for a participant in order to be regarded as cognitively normal.

Patients were excluded if they had taken H_2-receptor antagonists, proton pump inhibitors, antibiotics, bismuth compounds, or nonsteroidal anti-inflammatory drugs in the preceding 4 weeks. Patients were also excluded if they had undergone previous gastric surgery; were on anticoagulant therapy; were alcohol abusers; had allergy to penicillin and macrolides; had evidence of gastric cancer or other neoplasms; or had severe cardiac, pulmonary, kidney, or liver disease.

All patients and/or their relatives gave their informed consent and the study protocol was approved by the local ethics committee.

CSF samples of 5 mL were obtained through lumbar puncture using a 24-gauge needle with special care to avoid blood contamination. The sample was transferred immediately and stored in a freezer at -70°C until assay for anti-H. pylori IgG antibody (within 20–25 days).

Anti-H. pylori IgG antibody levels in the serum were also determined in all AD patients and controls. For this purpose, blood samples were collected on the day of surgery; samples were centrifuged at 3,000 g for 10 min to obtain serum, then aliquoted and stored at -70°C in the laboratory freezer until assay (within 20–25 days). This investigation included a total of 54 CSF and blood samples, 27 from AD patients and 27 from age-matched controls.

All patients underwent upper gastrointestinal endoscopy and presence of H. pylori infection was further documented on the basis of histological analysis of gastric mucosa biopsy.

Anti-H. pylori IgG Analysis

In the present study the concentrations of anti-H. pylori IgG in the CSF and the serum samples were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Enzywell DIESSE Diagnostica Senese, Siena, Italy). H. pylori serological status has already been described previously (Kountouras et al., 2001; Kountouras et al., 2002). The manufacturer's recommended cutoff value of 10 U/mL was used to define each patient's serological analysis as positive or negative.

The dilution of serum samples was 1:101 with sample buffer, whereas the dilution of CSF samples was 1:2 with sample buffer. In particular, 100 μL of the patient sample contained 50 μL of CSF diluted with the sample buffer (containing BSA and 0.09% (w/v) sodium azide). Intra-assay and interassay coefficients of variation were 4–8% and and 10–12%, respectively.

The expected value in the CSF of the normal population is negative. However, in our laboratory, we have established our own normal range. The

cutoff value to determine H. pylori-positive cases in the CSF, according to the anti-H. pylori-specific IgG by measured ELISA, was determined as follows: The mean of the corrected optical density (OD) values of the CSF samples (based on the negative ELISA assay in serum) was calculated and added to three times the standard deviation (SD). Those CSF samples with an OD greater than the mean of negative CSF samples plus 3 SDs were considered to be positive, while those with an OD less than the mean of negative CSF samples plus 3 SDs were considered negative. According to this method, a cutoff value of 1.87 U/mL was established. Patients with a value less than 1.87 U/mL were considered as H. pylori negative (Mitchell, Mascord, Hazell, & Daskalopoulos, 2001).

Statistical Analysis

Clinical data and anti-H. pylori IgG titers in serum and CSF were expressed as mean ± SD. For comparisons of the age (years) of the patients versus controls, the nonparametric Mann–Whitney *U*-test was used, whereas for sex analysis, the two-tailed Fisher's exact test was applied. The latter test was also used to assess the difference in H. pylori-positive cases in serum and CSF between the groups. Mann–Whitney *U*-test was applied to compare the anti-H. pylori IgG antibodies in CSF in groups A and B. Two independent samples' *t*-test was applied for the comparison of mean MMSE score between CSF anti-H. pylori IgG positive and negative cases. Significance was set at $p < 0.05$.

RESULTS

Demographic, clinical data, and levels of anti-H. pylori IgG antibodies in serum and in CSF are shown in Table 1. There was no difference among the study groups in age and sex.

Alzheimer's disease patients' mean performance in cognitive tests was as follows: MMSE: 18.03 ± 6.56, CAMCOG: 56.63 ± 22.44, FRSSD: 13.36 ± 6.31, NPI: 10.55 ± 8.44, GDS: 3.4 ± 2.6. Controls' mean score in cognitive tests was 28.51 ± 1.36 in MMSE and 1.74 ± 1.63 in FRSSD. As expected, controls performed better than AD patients in MMSE ($p < 0.001$) and FRSSD ($p < 0.001$).

The mean concentration of anti-H. pylori IgG antibodies in the CSF of patients with AD was 10.53 ± 12.54 U/mL, significantly higher than that observed in the CSF of age-matched control participants (8.63 ± 8.01 U/mL; $p = 0.047$).

Table 1. Demographic data and levels of anti-H. pylori (Hp) IgG antibodies in serum and cerebrospinal fluid (CSF)

Variable	Group A[a] ($n = 27$)	Group B[b] ($n = 27$)	p value
Age (years): mean ± SD	70.62 ± 6.66	72.57 ± 7.8	0.311
Sex (M:F)	12:15	19:8	0.098
Anti-Hp IgG, serum (mean ± SD) (U/mL)	30.44 ± 33.94	16.24 ± 5.77	$p < 0.05$
Anti-Hp IgG, CSF (mean ± SD) (U/mL)	10.53 ± 12.54	8.63 ± 8.01	$p < 0.05$

[a]Group A: Alzheimer's disease patients; [b]Group B: Cognitively normal controls; SD: Standard deviation.

As shown in Table 2, the presence of anti-H. pylori IgG antibodies in the CSF of patients with AD correlated with the degree of severity of the disease.

The mean concentration of anti-H. pylori IgG antibodies in the serum of patients with AD was 30.44 ± 33.94 U/mL, significantly higher than that observed in the serum of age-matched control patients (16.24 ± 5.77 U/mL; $p = 0.041$).

DISCUSSION

The early events underlying AD remain uncertain, although environmental factors may be involved. In this respect, the possibility that micro-organisms can cause AD has recently been addressed; infiltration of the brain by pathogens acts as a trigger or cofactor for AD, with Herpes simplex virus type 1 and Chlamydophila being implicated most frequently (Itzhaki, Wozniak, Appelt, & Balin, 2004; Robinson, Dobson, & Lyons, 2004). These pathogens may cause the neurological damage that results in AD by eliciting inflammation.

Table 2. Mini-mental state examination (MMSE) score in Alzheimer's disease (AD) patients related to cerebrospinal fluid (CSF) anti-H. pylori IgG (>1.87 U/mL) positivity

MMSE	CSF Anti-H. pylori IgG > 1.87 U/mL	CSF Anti-H. pylori IgG < 1.87 U/mL	p value
Mean ± SD	15.42 ± 6.92	20.76 ± 4.69	$p < 0.05$
>20 ($N = 10$)	2	8	$p < 0.05$
<20 ($N = 17$)	12	5	

SD: Standard deviation.

In this regard, an infection-based animal model demonstrated that following intranasal inoculation of BALB/c mice with Chlamydia pneumoniae, amyloid plaques/deposits consistent with those observed in the AD brain develop, thereby implicating this infection in the etiology of AD (Itzhaki et al., 2004).

The current series investigated for the first time the concentration of anti-H. pylori IgG antibodies in the CSF and serum of patients with AD and compared their levels with those of age-matched cognitively normal controls. Notably, the patients and control subjects were consecutive to eliminate the possibility of selection of groups, and if any bias in the selection may exist, this is universal for groups, thereby not affecting the results. The mean concentration of anti-H. pylori IgG in the CSF and serum of patients with AD was significantly greater than those found in the controls. Thus, the question arising from these results is, what is the importance of the increased prevalence of H. pylori IgG antibodies in the CSF and serum of patients with AD, and wether there is any role of this common infectious agent in the pathobiology of AD.

Apart from the cellular immune defective mechanisms playing an important role in the neurodegenerative process in AD (Kountouras et al., 2007), recent evidence also suggests that the possible presence of anti-neuronal antibodies and autoimmune mechanisms may be responsible for eliciting neuronal cell death in AD (D'Andrea, 2005). A key finding not only demonstrated the abnormal presence of anti-brain autoantibodies (Fernandez-Shaw, Marina, Cazorla, Valdivieso, & Vazquez, 1997) and human Igs (Bouras, Riederer, Kovari, Hof, & Giannakopoulos, 2005; D'Andrea, 2005) in the brain parenchyma of AD tissues, but, most notably, specific neurons that showed degenerative, apoptotic features contained these vascular-derived antibodies. In addition, subsequent studies detected classical complement components, C1q and C5b-9, in these Ig-positive neurons, which were also highly associated with reactive microglia over the Ig-negative neurons. It is possible that the mere presence of anti-neuronal autoantibodies in serum, whose importance had been previously dismissed, may be without pathological consequence (because of the "immunological privilege" of the brain, which excludes a direct access of Ig to the CNS under normal conditions) until there is a blood-brain barrier dysfunction to allow the deleterious effects of these autoantibodies access on their targets. These findings suggest autoimmunity-induced cell death in AD (Bouras et al., 2005; D'Andrea, 2005). The evidence that autoantibodies may contribute to neuronal cell death in AD is also consistent with a wider literature implicating a causative role for autoantibodies in many peripheral neuropathies including Guillain–Barré syndrome (Kountouras et al., 2005) that share pathogenetic similarities with AD as well as glaucomatous optic

neuropathy defined as "ocular AD"; the autoantibodies directed toward retinal antigens may be involved in facilitating apoptotic cell death in glaucoma patients (Kountouras et al., 2007; Maruyama, Nakazawa, & Ohguro, 2001).

The association between H. pylori infection and gastric autoimmunity is well established (Parente et al., 2001). It is relevant to note that H. pylori infection is associated with the synthesis of parietal cell autoantibodies, that cross-react with the gastric mucosa and, after eradication of the infection, persist and contribute to recurrent antral chronic gastritis and intestinal metaplasia (Kountouras et al., 2005). Moreover, serum parietal cell autoantibodies correlate with anti-H. pylori antibody titers (Basso et al., 2000). Therefore, the serological titer of anti-H. pylori seems to reflect the autoimmunity status that correlates with gastric mucosal atrophy, thereby indirectly offering evidence of the severity of histologic inflammatory changes (Sheu et al., 1997).

Remarkably, molecular mimicry of host structures by the saccharide portion of lipopolysaccharides of the gastrointestinal pathogens Campylobacter jejuni (C. jejuni) and H. pylori are thought to be connected with the development of autoimmune sequelae observed in neuropathies. C. jejuni, a principal cause of gastroenteritis, is the most frequent antecedent infection in Guillain–Barré syndrome, an inflammatory neuropathy. In addition, 46% of patients with Guillain–Barré syndrome have specific IgG antibodies to VacA of H. pylori in the CSF, and the sequence homology found between VacA and human [Na(+)/K(+)] ATPase A subunit suggests that antibodies to VacA involve ion channels in abaxonal Schwann cell plasmalemma leading to demyelination in some patients within the CSF (Chiba et al., 2002).

In view of the previously mentioned data, it is possible to suggest that the increased titer of anti-H. pylori IgG antibodies observed in the CSF and serum samples of patients with AD, may indirectly offer evidence for a role of H. pylori in the cascade events of neurodegenerative process in AD. A similar speculation may be also applied in glaucomatous optic neuropathy (Kountouras et al., 2005). The most likely mechanism for the role of this agent is via molecular mimicry autoimmune sequelae. An interesting, although rather gross correlation, is the observation that the positivity status for H. pylori in the CSF appeared to correlate with the severity of clinical status in AD patients. Nevertheless, further studies in large AD cohorts throughout the clinical range and utilizing all AD parameters are needed to support the hypothesis that the presence of IgG antibodies to H. pylori may adversely influence progression of AD.

Notably, the possibility of the passive passage of IgG and antibodies through a normal blood-CSF barrier that might explain our findings should not be excluded. Moreover, theoretically, in case of advanced AD, with cerebral

atrophy, the blood-CSF barrier might be frequently altered with higher CSF protein content and higher transudation of IgG and antibodies in the CSF, thereby might, again, explaining the correlation of the positivity status for H. pylori with the severity of clinical status in our AD patients. However, this consideration seems to be unlikely, because blood-brain barrier dysfunction is found early in the disease before the onset of clinical dementia (Skoog et al., 1998), and moreover, the permeability of the blood-CSF barrier does not correlate with dementia severity (Hampel, Kotter, & Moller, 1997).

In this regard, some limitations of the present study may include the relatively small number of patients, and that the CSF antibody titers were not normalized to another protein to serve as control, such as IgG or albumin.

The expression of two H. pylori proteins, cagA and vac A is considered to be connected with pathogenicity of this bacterium. In this respect, H. pylori has been implicated in numerous extradigestive conditions (de la Torre, 2006; Gasbarrini et al., 1999; Grau et al., 2001; Miklossy, 2003; Singh et al., 2002) and there is evidence of a possible role particularly of CagA-positive H. pylori infection in some of these conditions including iron-deficiency anemia and ischemic heart disease (Sokić-Milutinović, Todorović, & Milosavljević, 2004). Because we did not investigate the possible pathogenetic role of CSF CagA and/or Vag A cytotoxins in AD or other neurodegenerative diseases, future relative studies are needed to elucidate this field.

It is tempting to speculate that H. pylori infection may negatively influence the neurodegerative process in AD. Amongst the possible mechanisms involved may be (a) molecular mimicry-related toxicity by H. pylori autoantibodies and various H. pylori toxic antigens to endothelium and neuron proteins; (b) release in the circulation of H. pylori-related vasoactive substances, or (c) increased susceptibility of neurons to H. pylori various substances. These hypotheses require future validation. In theory, H. pylori antibodies may circulate in the bloodstream and enter the CSF via the blood-CSF barrier where they may reach a level sufficient to impact the development or progression of AD.

In conclusion, our data suggest that AD appears to have an infectious link related to H. pylori involved in the pathophysiology of this disease.

Declaration of Interest

The authors report no conflicts of interest.

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ORIGINAL COMMUNICATION

Eradication of *Helicobacter pylori* may be beneficial in the management of Alzheimer's disease

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Abstract Infectious agents have been proposed as potential causes of Alzheimer's disease (AD). Recently, we documented a high prevalence of *Helicobacter pylori* (*Hp*) infection in patients with AD. We aim to access the effect of *Hp* eradication on the AD cognitive (MMSE: Mini Mental State Examination and CAMCOG: Cambridge Cognitive Examination for the Elderly) and functional (FRSSD: Functional Rating Scale for Symptoms of Dementia) status parameters. In the first part of the study, a total of 50 consecutive patients with AD and 30 age-matched anaemic controls underwent an upper gastrointestinal endoscopy, and gastric mucosal biopsies were obtained to detect the presence of *Hp* infection by histologic analysis and rapid urease test. Serum anti-*Hp*-specific IgG level was analysed by enzyme-linked immunosorbent assay. In the second part, *Hp*-positive AD patients received a triple eradication regimen (omeprazole, clarithromycin and amoxicillin), and all patients were followed up for 2 years, while under the same treatment with cholinesterase inhibitors. *Hp* was detected in 88% of AD patients and in 46.7% of controls ($P < 0.001$). *Hp* eradication was successful in 84.8% of treated patients. At the 2-year clinical endpoint, cognitive and functional status parameters improved in the subgroup of patients where *Hp* eradication was successful ($P < 0.001$ and $P = 0.049$ for MMSE and CAMCOG, respectively; $P < 0.001$ for FRSSD), but not in the other patients. *Hp* eradication may positively influence AD manifestations, suggesting a possible common link between *Hp* and AD.

Keywords Alzheimer's disease · *Helicobacter pylori* · Histologic analysis · Rapid urease test

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Introduction

Alzheimer's disease (AD) is by far the most common cause of dementia of ageing [18]; it is a horribly debilitating disease that will increase in prevalence as the populations of the USA and Europe continue to age. The disease is characterised by progressive impairment in memory, visuospatial skills, complex cognition, language and personality in its earlier stages.

In general, cognitive performance in ageing individuals is frequently adequate until the individual suffers a challenge, including severe infection; systemic infections are vulnerable to cognitive decline, and pharmacologic strategies to decrease neuroinflammation associated with infection might be important for reducing neurobehavioural deficits in the elderly [3, 10]. Specifically, although

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the early events underlying AD remain uncertain, the consideration that microorganisms can cause AD has recently been addressed [15, 19, 46]; infiltration of the brain by pathogens acts as a trigger or co-factor for AD, with *Herpes simplex* virus type 1 (HSV1) and *Chlamydophila (Chlamydia) pneumoniae* being most frequently implicated [15, 46]. These pathogens may cause the neurological damage that results in AD by eliciting inflammation. In this regard, an infection-based animal model demonstrates that following intranasal inoculation of BALB/c mice with *Chlamydia pneumoniae*, amyloid plaques/deposits consistent with those observed in the AD brain develop, thereby implicating this infection in the aetiology of AD [15].

Helicobacter pylori (*Hp*) is a gram-negative, spiral, flagellated bacterium, comprising more than 1,400 genes, that colonises the gastric mucosa of most humans worldwide, mainly affecting older adults in the developed world, including Greece [43]. It is associated with various upper gastrointestinal (GI) diseases [6, 43] and has also been implicated in a variety of extradigestive vascular conditions, including ischaemic heart disease [35], ischaemic cerebrovascular [33] and functional vascular disorders caused by vascular dysregulation, frequently detected in AD.

The association of *Hp* infection (*Hp*-I) and AD has also only recently been addressed by three studies [24, 26, 31]. A higher seropositivity for anti-*Hp* IgG antibodies was reported in patients with AD than in age-matched controls, but this serological test has limitations because it does not discriminate between current and old infections [24]. Such a distinction is essential because current *Hp*-I induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves [25, 26], thereby affecting or perpetuating neural tissue damage. Moreover, eradication of *Hp*-I might delay AD progression, particularly at early disease stages, including mild cognitive impairment (MCI). Based on the histological analysis of gastric mucosa biopsy for the documentation of *Hp*-I, Kountouras and associates [24] reported a higher prevalence of *Hp*-I in patients with AD than in age-matched controls, accompanied by increased homocysteine (Hcy) concentration, an independent risk factor for dementia and AD, thereby suggesting an association between these two diseases. The authors also reported similar data in patients with MCI compared with age-matched controls [26]. Demonstrating the association of *Hp* and AD and proving the benefit of eradicating *Hp*-I in the clinical course of the disease may have a major impact on its treatment. However, before antibiotic therapy for *Hp*-I becomes an established step in the management of AD, sufficient evidence must be provided that AD parameters are positively influenced by the eradication of *Hp*-I.

The objective of this series was to evaluate the effect of *Hp* eradication on cognitive and functional status parameters of patients with AD. We have therefore designed methods to confirm and quantify our hypothesis that *Hp* eradication therapy has a beneficial effect on these AD parameters in *Hp*-positive patients with AD.

Materials and methods

Patients

This was a two-part series. Part 1 was designed to evaluate the prevalence of *Hp*-I infection in AD. Fifty consecutive patients with documented AD and 30 age-matched anaemic controls were included in this part of the study.

All participants had been referred to the Memory and Dementia Outpatient Clinic by their caregivers, mainly relatives, who certified a cognitive deterioration and/or other cognitive functional disturbances in the participants for at least a period of 6 months. Patients were diagnosed with probable Alzheimer's dementia according to the NINCS-ADRDA and DSM-IV criteria [34]. Screening procedure for their evaluation was conducted at their first visit to the Memory and Dementia Outpatient Clinic. Patients and controls underwent neuropsychological assessment that included measurement for cognitive deterioration (MMSE: Mini Mental State Examination and CAMCOG: Cambridge Cognitive Examination for the Elderly), functional disorders (FRSSD: Functional Rating Scale for Symptoms of Dementia), neuropsychological disorders (NPI: Neuropsychiatric Inventory) and depression (GDS: Geriatric Depression Scale, HDRS: Hamilton's Depression Rate Scale). The aforementioned scale battery required 90 min on average. MMSE was used as a screening test for cognitive deterioration, assessing orientation in time and place, naming, repetition, immediate and late recall, ideational apraxia and constructional praxis (total score 30). CAMCOG was used for a more thorough investigation of the above-mentioned cognitive functions (total score 107). FRSSD assessed patients' ability to carry out routine tasks such as eating, dressing and toileting (total score 42). FRSSD total score over seven revealed functional difficulties in everyday living, while total score 5–7 was regarded as borderline. The twelve-item NPI was the only scale provided to the caregiver assessing patient's neuropsychiatric symptoms, such as hallucinations, apathy-indifference, aggressiveness, sleep and eating disorders and depression. Both frequency (scale 1–4) and severity (scale 1–3) of every disorder was measured (total score 120). Depression being the major cause of dementia was regarded as an exclusion criterion. It was estimated through scales of GDS (total score 15, cutoff 5) and HDRS (total

score 55, cutoff 16), both provided by the patients themselves. Neuropsychological assessment was conducted by the same neuropsychologist for all patients. Apart from the above-mentioned assessment, MRI tomography was conducted as diagnostic neuroimaging technique in order to confirm temporal lobe and hippocampal formation atrophy. It was also used in order to exclude other causes of dementia (stroke, tumour, fronto-temporal dementia, etc.). Patients with vascular, Lewy-body, fronto-temporal and other types of dementia were excluded from the study. We also excluded patients with known or subclinical thyroid disorders as well as patients with depression. None of the patients had previously been treated with cholinesterase inhibitors (ChEIs), memantine or any other pharmacological treatment for dementia.

Inclusion of cognitively normal controls required MMSE score >24, CAMCOG score >85 and FRSSD score <5. Moreover, in addition to scores above cutoff in cognitive tests, subjective memory complaints should be absent for a control participant in order to be regarded as cognitively normal.

All patients and controls underwent diagnostic upper GI endoscopy after informed consent. Apart from upper GI endoscopy, the control subjects underwent lower endoscopy to investigate mild iron-deficiency anaemia; the GI mucosa appeared to be without obvious macroscopic abnormalities. Participants were excluded if they had taken H_2-receptor antagonists, proton pump inhibitors, antibiotics, bismuth compounds or nonsteroidal anti-inflammatory drugs in the preceding 4 weeks. Participants were also excluded if they had undergone previous gastric surgery; received anticoagulant therapy; were alcohol abusers; had allergy to penicillin or macrolides; had gastric cancer or other neoplasms; or had severe cardiac, pulmonary, kidney or liver disease.

All participants and/or their relatives signed a consent form prior to enrollment, and the study protocol was approved by the local ethics committee. All patients received the same ChEI during the 2-year follow-up period of the study. None of the participants in this study received oral drugs that could influence cognitive state, other than the medication prescribed by the researchers.

In the second part of the study, 56 *Hp*-positive patients with AD (40 patients from the first part of the study and 16 new AD patients) were included. The additional 16 patients (11 female, mean age ± SD 74 ± 6.83, range 60–87 years) were selected according to the same aforementioned inclusion criteria. Therefore, 61 patients with AD (56 *Hp*-positive and 5 *Hp*-negative patients at baseline who did not receive eradication treatment) were subsequently observed in the second part of the study, which evaluated the effect of administration of *Hp* eradication regimen on cognitive and functional status parameters over a 2-year follow-up period (Fig. 1).

According to standard clinical practice in Europe, the *Hp* eradication regimen comprised a 1-week course

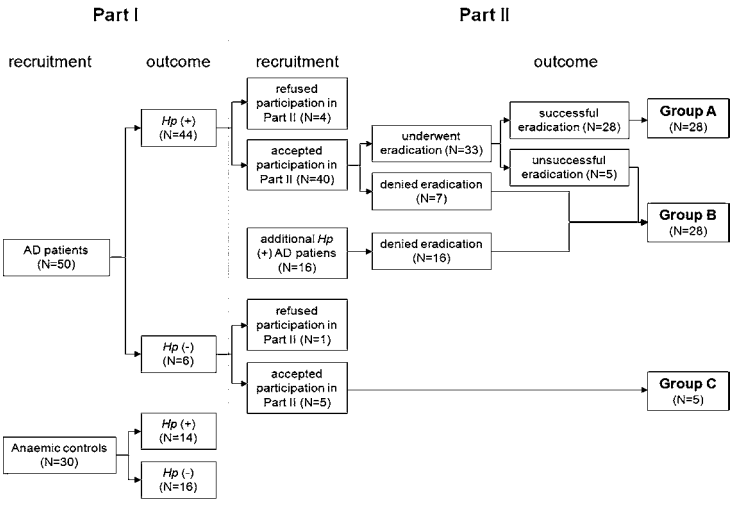

Fig. 1 Participants' flow chart showing the detailed study design. Group A included AD patients for whom eradication treatment was successful ($N = 28$). Group B included 5 AD patients from Part I for whom eradication therapy had failed, 7 AD patients from part I who denied eradication therapy, as well as 16 newly recruited *Helicobacter pylori* (*Hp*)-positive AD patients who did not undergo eradication therapy ($N = 28$). Group C included AD patients *Hp*-negative from part I ($N = 5$)

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consisting of omeprazole (20 mg bid), clarithromycin (500 mg bid), and amoxycillin (1 g bid), followed by omeprazole 20 mg once daily for 1 month [12].

Study design

Hp detection methods were described previously [21]. Biopsy urease test and histopathology process were also described previously [21]. Success of the *Hp* eradication regimen was evaluated by control endoscopy at least 8 weeks after cessation of therapy, and patients were considered as *Hp* negative if both histology and the rapid urease test proved negative. The neuropsychologist assessing cognitive and functional state in this study was masked to the *Hp* status of the patients.

The follow-up study population was classified into three AD groups: patients for whom *Hp* eradication treatment was successful (group A); those for whom eradication of *Hp* had failed, and they refused and/or were noncompliant with their eradication therapy (group B); and those who were *Hp* negative at baseline (group C, Fig. 1).

Statistical analysis

Mann–Whitney *U*-test, Fisher's exact test, chi-square, odds ratios, 95% CI, two-tailed *t*-test and one-way analysis of variance were used. Significance was set at $P < 0.05$. The analysis was performed by using the statistical software package SPSS (Statistical Package for Social Sciences, version 13.0; SPSS Inc., Chicago, IL).

Results

The patients with AD had a higher prevalence of *Hp*-I than controls, as verified by the histologically confirmed presence of *Hp* in 44 of 50 (88%) AD cases, including 14 patients who tested negative in the gastric mucosa urease test, and in 14 of the 30 (46.7%) control participants ($\chi^2 = 14.1$, $P < 0.001$; Table 1). The odds ratio for the association of *Hp* with AD was 8.4 (95% CI, 2.4–28.7). The mean serum IgG anti-*Hp* level was also significantly higher in patients with AD (34.0 ± 40.1 U/ml) than in controls (17.0 ± 18.1 U/ml; $P = 0.016$). The AD patients exhibited histologically confirmed multifocal (body and antral) gastritis more often than controls (49/50 vs. 21/30; $P < 0.001$).

Outcome of Hp eradication therapy

One *Hp*-negative and four *Hp*-positive patients finally refused to participate and were excluded from the second part of the study. Of the remaining 40 *Hp*-positive AD patients, 33 received and 7 refused to receive eradication therapy. *Hp* eradication was successful in 28 of these 33 (84.8%) patients (group A, Fig. 1). Treatment was unsuccessful in the remaining five patients; apart from the 7 *Hp*-positive AD patients who refused to receive eradication therapy, 16 additional *Hp*-positive patients were recruited in the second part of the study who also refused to receive eradication treatment, thereby remaining *Hp*-positive throughout the follow-up period (group B, Fig. 1). All group A patients were compliant with their eradication therapy as determined by the number of tablets and capsules remaining after therapy. Adverse effects were generally mild, including mild abdominal pain, occasional nausea or vomiting, diarrhoea and stomatitis. None of the patients discontinued their therapy because of these mild adverse effects.

When compared with baseline values (49.3 ± 30.4 U/ml), the mean serum IgG anti-*Hp* level was significantly reduced in group A patients at 3-month follow-up (24.5 ± 12.9 U/ml) ($P < 0.001$). In group B patients, this parameter had increased at 3-month follow-up (32.6 ± 14.7 U/ml at 3 months vs. 28.2 ± 11.2 U/ml baseline; $P = 0.03$). In group C patients, who did not receive *Hp* eradication therapy, both mean serum IgG anti-*Hp* values at baseline and at 3 months were within normal limit levels (6.7 ± 2.4 U/ml at 3 months vs. 7.1 ± 2.1 U/ml baseline; $P = 0.04$).

Table 1 *Helicobacter pylori* positivity in patients with Alzheimer's disease and anaemic controls

Characteristic AD	Patients ($n = 50$)	Controls ($n = 30$)	Odds ratio (95% CI)	*P* value
Mean ± SD age (range), *years*	65.0 ± 6.9 (53–80)	62.2 ± 8.6 (44–70)	NA	NS
Sex (M/F)	18/32	14/16	NA	NS
Positive urease test (gastric mucosa)	30 (60%)	14 (46.7%)	1.7 (0.7–4.3)	NS
Mean ± SD serum anti *H. pylori* IgG (U/ml)	34.0 ± 40.1	17.0 ± 18.1	NA	0.016
Histologically confirmed presence of *H. pylori*	44 (88%)	14 (46.7%)	8.4 (2.4–28.7)	<0.001

Unless otherwise indicated, data are number (percentage) of patients

CI confidence interval, *NA* indicates not applicable

Outcome of AD parameters

Baseline Mean Mini Mental State Examination (MMSE) ($F = 0.986$, $df_{wg} = 60$, $df_{bg} = 2$, $P = 0.379$), Cambridge Cognitive Test (CAMCOG) ($F = 1.238$, $df_{wg} = 60$, $df_{bg} = 2$, $P = 0.302$) and Functional Rating Scale for Symptoms of Dementia (FRSSD) ($F = 1.646$, $df_{wg} = 60$, $df_{bg} = 2$, $P = 0.202$) scores did not differ significantly among the three groups.

Table 2 and Figs. 2, 3, and 4 show the AD parameters for groups A–C at baseline and 1 and 2 years after treatment. At the treatment endpoints selected in the study (1 and 2 years), a significant improvement was found in patients' cognitive and functional status parameters in group A compared with baseline readings. In contrast, the same parameters deteriorated from baseline to 1- and 2-year follow-up in group B. AD parameters did not differ or slightly deteriorated (not statistically significant) from baseline to 1- and 2-year follow-up values in group C.

Table 2 Comparison of mean Mini Mental State Examination (MMSE), Cambridge Cognitive Examination for the Elderly (CAMCOG) and Functional Rating Scale for Symptoms of Dementia (FRSSD) parameters for all patients with Alzheimer's disease at baseline and after 1 and 2 years of follow-up

Patient group[a]	Mean ± SD measurement value		
	Baseline	1-Year	2-Year
MMSE			
A	17.46 ± 6.09	19.6 ± 6.08	19.92 ± 5.94
B	17.07 ± 6.15	14.39 ± 7.1	11.25 ± 5.87
C	21.2 ± 5.63	17.8 ± 9.67	16.4 ± 11.01
CAMCOG			
A	52.07 ± 24.3	57.71 ± 25.49	62.86 ± 22.42
B	55 ± 17.58	46.4 ± 19.98	38.3 ± 15.55
C	65.5 ± 14.84	56 ± 35.35	53.33 ± 25.38
FRSSD			
A	12.53 ± 6.81	10.38 ± 5.98	9.15 ± 6.52
B	15.32 ± 8.15	18.6 ± 7.59	21.53 ± 8.21
C	10.2 ± 2.48	10 ± 4.06	17.2 ± 8.98

Patient group[a]	MDM (95% CI)	*P* value
Change from baseline at 1 year		
MMSE		
A	2.14 (2.82 to 1.46)	<0.001
B	−2.67 (−1.55 to −3.8)	<0.001
C	−3.4 (2.05 to −8.85)	0.159
CAMCOG		
A	5.64 (8.7 to −2.58)	0.002
B	−8.6 (−4.38 to −12.81)	0.001
C	−9.5 (174.74 to −193.73)	0.631
FRSSD		
A	−2.15 (−1.05 to −3.24)	<0.001
B	3.28 (5.01 to 1.54)	0.001
C	−0.2 (4.47 to −4.87)	0.911
Change from baseline at 2 years		
MMSE		
A	2.46 (3.28 to 1.64)	<0.001
B	−5.82 (−4.68 to −6.95)	<0.001
C	−4.8 (2.35 to −11.95)	0.136
CAMCOG		
A	5.13 (10.23 to −0.028)	0.049
B	−18.3 (−16.27 to −20.32)	<0.001
C	−17.66 (17.84 to −53.17)	0.166
FRSSD		
A	3.38 (−2.1 to −4.66)	<0.001
B	6.15 (8.92 to 3.38)	<0.001
C	7 (19.19 to −5.19)	0.186

MDM mean difference of the means, *CI* confidence interval

[a] Group A includes only patients in whom *Helicobacter pylori* (*Hp*) was successfully eradicated; group B, patients in whom the *Hp* eradication regimen was unsuccessful and/or denied to receive *Hp* eradication treatment; group C, *Hp*-negative patients at baseline; n indicates the number of patients treated

Discussion

In the first part of this series, by documenting a higher prevalence of *Hp*-I in an AD cohort compared with an age- and sex-matched control group, we established for the first time a significant relationship between *Hp*-I infection and AD [24]. *Hp*-I was determined by histologic detection of organisms in mucosal biopsy specimens, considered as the actual gold standard for the diagnosis of this infection. A higher seropositivity for anti-*Hp* IgG antibodies was also reported in 30 patients with AD than in age-matched controls [31].

It is important to consider whether the rate of *Hp*-I in the control group has been negatively influenced by the coexistence of anaemia. Existing data show that anaemia does not protect against *Hp* development. Anaemic controls have been used before [41], and the frequency of *Hp*-I in the anaemic control group matches that of the general population in Greece and that reported for other ethnic populations [50]. Moreover, it is unlikely that individuals with iron-deficiency anaemia are protected against *Hp*-I because it is thought that the infection is actually associated with iron- and/or vitamin B_{12}-deficiency anaemia [13]. In addition, eradication of *Hp*-I may be associated with reversal of iron and/or vitamin B_{12} deficiency and improvement in anaemia [16].

In the second part of the study we obtained an acceptable eradication rate of 84.8%. Similar eradication rates have been achieved by others [43]. Moreover, *Hp* eradication was beneficial for the cognitive and functional state in patients in whom *Hp* was successfully eradicated (group A), thereby possibly altering the progressive nature

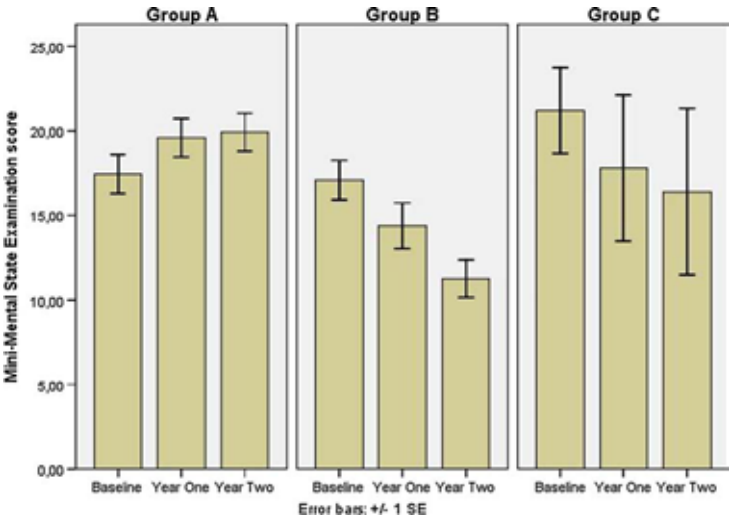

Fig. 2 Mean Mini Mental State Examination scores at baseline and 1 and 2 years after treatment in patients for whom *Helicobacter pylori* (*Hp*) treatment was successful (group A); those for whom eradication of *Hp* failed and/or those who denied to receive *Hp* eradication treatment (group B); and those who were *Hp*-negative at baseline (group C). Error bars indicate standard error (SE)

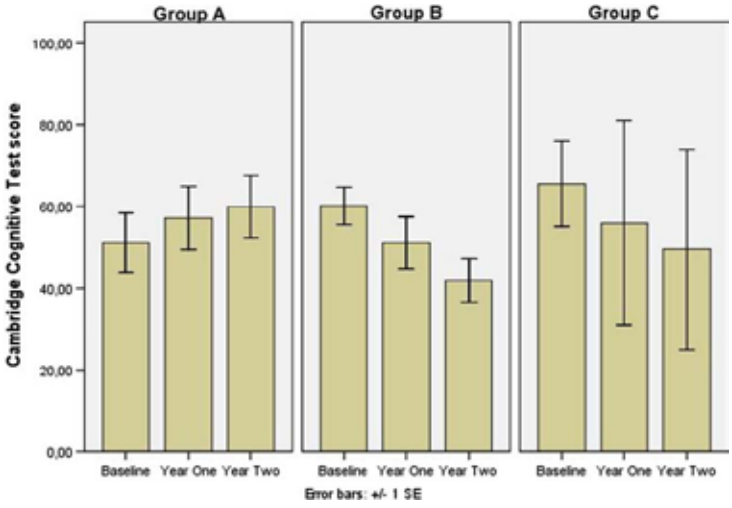

Fig. 3 Mean Cambridge Cognitive Examination for the Elderly scores at baseline and 1 and 2 years after treatment in patients for whom *Helicobacter pylori* (*Hp*) treatment was successful (group A); those for whom eradication of *Hp* failed and/or those who denied to receive *Hp* eradication treatment (group B); and those who were *Hp*-negative at baseline (group C). Error bars indicate standard error (SE)

of AD and increasing survival of AD patients. This might not be the case in groups B and C despite the fact that all groups were maintained on the same ChEI they received at baseline. In this regard, parameters significantly associated with reduced survival at diagnosis include mainly increased severity of cognitive impairment and decreased functional level strongly related with shortened survival [4, 28]. Longer term follow-up, however, is required to evaluate the possible beneficial effect of *Hp* eradication therapy.

The results of this study suggest that eradication therapy may somehow improve the degenerative process in AD. It

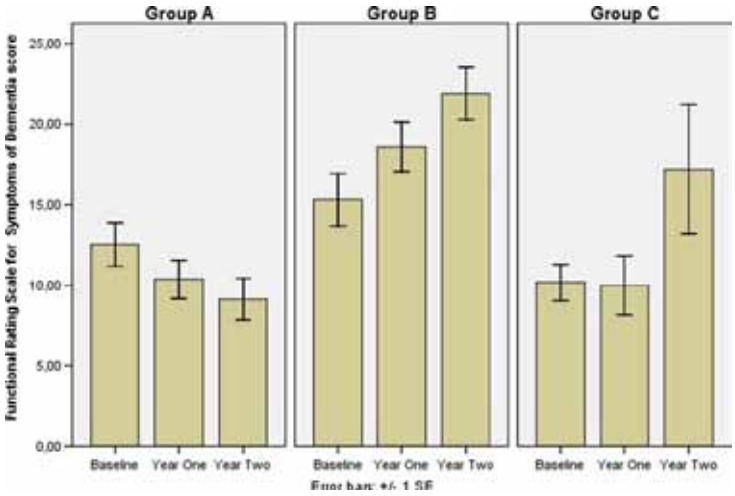

Fig. 4 Mean Functional Rating Scale for Symptoms of Dementia scores at baseline and 1 and 2 years after treatment in patients for whom *Helicobacter pylori* (*Hp*) treatment was successful (group A); those for whom eradication of *Hp* failed and/or those who denied to receive *Hp* eradication treatment (group B); and those who were *Hp*-negative at baseline (group C). Error bars indicate standard error (SE)

should be noted, however, that the number of patients in the groups, particularly in group, C was small, and it may not, therefore, be possible to draw definitive conclusions. Future studies are needed to focus on the influence of *Hp* on the degenerative process in the brain. It is conceivable that *Hp* induces relative mechanisms and/or agents such as the synthesis of various mediators (e.g., cytokines), which may be detrimental to the degeneration of the demented brain. Specifically, there is evidence for abnormal cellular immune and apoptotic mechanisms playing an important role in the *Hp*-associated GI pathologies and potentially affecting the neurodegenerative process in AD. Interestingly, molecular mimicry of host structures by the saccharide portion of lipopolysaccharides of the GI pathogens *Campylobacter jejuni* (*C. jejuni*) and *Hp* is thought to be connected with the development of autoimmune sequelae observed in neuropathies. *Campylobacter jejuni*, a principal cause of gastroenteritis, is the most common antecedent infection in Guillain-Barré syndrome (GBS), an inflammatory autoimmune neuropathy sharing comparable pathogenic mechanisms with AD [23]. Chemical analyses of the core oligosaccharides of neuropathy-associated *C. jejuni* strains have revealed structural homology with human gangliosides. Serum antibodies against gangliosides are found in one-third of patients with GBS, but are generally absent in enteritis cases. Collective data suggest that the antibodies are induced by antecedent infection with *C. jejuni*, and subsequently react with nerve tissue, causing damage [40], possibly by apoptosis. In addition, 46% of patients with GBS have specific IgG antibodies to VacA of *Hp* in the cerebrospinal fluid, which are probably associated with some components of the peripheral nerve myelin, thereby showing considerable demyelination of the peripheral nerves; the sequence homology found between 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 these patients [5, 27]. In this regard, it is relevant to speculate that such anti-*Hp*-mediated apoptotic mechanisms might also lead to degeneration of ganglion cells, thereby contributing to AD neuropathy or other degenerative neuropathies, such as glaucoma, defined as ocular AD. Support for this theory is provided by our observations indicating that the titre of anti-*Hp* IgG antibodies in the aqueous humour of patients with glaucoma may reflect the severity of glaucomatous damage [22].

The question raised is how exactly *Hp*-I influences the pathophysiology of AD. This bacterium may be involved in the pathophysiology of AD by one of the following mechanisms: (1) Promoting platelet and platelet–leukocyte aggregation [24, 26]. Platelet activation and aggregation have also been proposed to play possible pathophysiologic roles in the development of AD [47]. (2) Inducing chronic atrophic gastritis with a concomitant decrease in vitamin B12 and folate concentrations, thereby increasing the concentration of Hcy, an independent risk factor for AD and a potent contributor to vascular disorders implicated in endothelial damage and neurodegeneration via oxidative injury. Specifically, an increased serum Hcy concentration has been shown in our AD patients with *Hp*-I [24]. Chronic

gastritis, as a result of *Hp*-I, can lead to malabsorption of vitamins B_{12} and folate, which results in failure of methylation by 5-methyl-tetrahydrofolic acid and hence accumulation of Hcy [24]. The elevated Hcy, in turn, could trigger endothelial damage and neurodegeneration via oxidative injury and result in atherothrombotic disorders and AD. (3) Releasing large amounts of proinflammatory and vasoactive substances, such as cytokines [interleukin (IL)-1, IL-6, IL-8, IL-10, IL-12, tumour necrosis factor (TNF)-α, interferon-γ], eicosanoids (leucotrienes, prostaglandins catalysed by cyclo-oxygenase enzymes) and acute phase proteins (fibrinogen, C-reactive protein) [24, 25], involved in a number of vascular disorders possibly including AD and other AD-related neuropathies such as glaucoma [25]. (4) Stimulating mononuclear cells to produce a tissue factor-like procoagulant that converts fibrinogen into fibrin [25]. (5) Causing the development of cross mimicry between endothelial and *Hp* antigens. (6) Producing reactive oxygen metabolites (ROMs) and circulating lipid peroxides [24, 25], which have been implicated in the pathophysiology of AD [31, 45]; accumulating evidence suggests that ROMs are potent deleterious agents causing cell death or other forms of irreversible cell damage, and oxidative stress participates in the neuronal loss in AD [25, 48]. ROMs accumulation impairs endothelial barrier function and promotes leucocyte adhesion, induces alterations in normal vascular function and might result in the development of AD [1], events that are also triggered in *Hp*-induced GI injury [20]. (7) Influencing the apoptotic process that plays a potential role in the pathogenesis of many neurodegenerative diseases including AD, glaucomatous neuropathy [24, 25] or Down syndrome; the latter predisposes to the early onset of the neurodegeneration of AD [12]. In particular, increased endothelin-1 (a potent constrictor of arterioles and venules), nitric oxide (NO) and inducible nitric oxide synthase (iNOS) levels are associated with *Hp*-I [21]. Relevant data in AD indicate that endothelin-1-like immunoreactivity in the AD brains is significantly increased in the frontal and occipital cortex compared to those in control brains, thereby explaining the decreased cerebral blood flow in AD patients [39]. Besides, recent evidence in humans indicates that the expression of the nitrergic system, the synthesis of NO, the peroxynitrite reactive production and protein tyrosine nitration are activated over the entire course of AD, and that the presence of amyloid-beta peptide (Abeta) increases the presence of neuronal nitric oxide synthase (nNOS) and iNOS isoforms over the course of AD in pyramidal-like neurones [7]; the overproduction of NO, the increase in both peroxynitrite and superoxide production, the mitochondrial membrane depolarisation and the caspase activation contribute to neuronal death [7, 17], mainly via apoptosis. NO is a rapidly diffusing gas and a potent neurotoxin that may facilitate the apoptotic death of retinal ganglion cells in glaucomatous optic neuropathy [11, 51], and probably in AD neuropathy [7, 32]. Further studies, however, are needed to clarify the aforementioned points.

Finally, a few shortcomings need to be addressed in our series. In contrast to data presented for other intracellular infectants [*Chlamydia pneumoniae*, *Herpes simplex virus* (HSV)-1, *Borrelia* species] found in brain regions demonstrating considerable AD pathology [2, 14, 30, 37, 38, 42], most of the aforementioned mechanisms of how *Hp* infection could influence the pathophysiology of AD are not necessarily specific to the regional pathology observed in the AD brain, nor is there a specific temporal sequence indicated by which *Hp* infection would directly injure the brain, at the present time. However, *Hp*, an extracellular bacterium, could affect the brain and other target organs, such as the heart, indirectly, through the release of numerous cytokines, including TNF-α acting at a distance. TNF-α and IL-6 (TNF-α is the main trigger for the production of IL-6 by a variety of cells) play important roles in the regulation of the synthesis of other acute phase proteins, which are established risk factors for atherosclerosis, such as fibrinogen and factor VIII. These cytokines also have profound effects on lipid metabolism directly at the site of the atherosclerotic lesion, but could influence the atheroma process through blood circulating levels, distant production of cytokines or through stimulating circulating white blood cells to produce them, thereby contributing to the pathogenesis of brain diseases including AD [11, 36, 49].

In addition, a *Hp* eradication regimen might have influenced other infections in the AD, particularly those of *Chlamydia peneumoniae* and *Borrelia* species, and could explain, at least partly, the positive improvement observed in our AD patients at the post-treatment period. However, the prevalence of *Chlamydia peneumoniae* and possibly *Borrelia* species infections are very low in both child and adult Greek populations [44, 52, 53], and, moreover, our participants did not report or present any characteristic clinical features suggesting the aforementioned infectious diseases.

Besides, we did not investigate the APOE gene (NCBI Entrez gene 348), the strongest genetic risk factor for later onset AD [54]. In this respect, there is evidence suggesting that some relationship exists between the APOE4 gene product and the pathobiology of the intracellular *Chlamydophila* (*Chlamydia*) *pneumoniae* involved in AD pathogenesis [8, 9]. Moreover, the risk of developing AD is much greater in patients HSV-1-positive in brain who also possess an APOE4 allele than in those with only one of these factors [29]. Therefore, future relative studies might clarify the potential relationship between this gene and the pathobiology of the extracellular *Hp* species.

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Implications for a role of interleukin-23 in the pathogenesis of chronic gastritis and of peptic ulcer disease

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Summary

The present study aimed to investigate the role of gastric mucosa for the secretion of interleukin (IL)-23 in chronic gastritis. One hundred and one patients were enrolled; 47 with duodenal ulcer, 33 with gastric ulcer and 31 with chronic gastritis. Biopsies were incubated in the absence/presence of endotoxins. Supernatants were collected and IL-23 and IL-1β were measured by enzyme-linked immunosorbent assay. Scoring of gastritis was performed according to the updated Sydney score. Patients with duodenal and gastric ulcer and those with chronic gastritis had similar scores of gastritis. IL-23 was higher in supernatants of tissue samples of *Helicobacter pylori*-positive than of *H. pylori*-negative patients. No differences were recorded in concentrations of IL-23 and IL-1β between patients with duodenal ulcer, gastric ulcer and chronic gastritis. Positive correlations were found between IL-23 of patients with both duodenal and gastric ulcer and chronic gastritis and the degree of infiltration of neutrophils and monocytes. Similar correlations were observed between IL-23 and IL-1β. IL-23 secreted by the gastric mucosa could be implicated in the pathogenesis of chronic gastritis. IL-23 was released in the presence of *H. pylori* from the inflamed gastric mucosa and followed the kinetics of IL-1β.

Keywords: chronic gastritis, gastric mucosa, IL-23, proinflammatory cytokines

Introduction

Chronic gastritis occurs in the setting of infection by *Helicobacter pylori*. *H. pylori*-associated gastritis is characterized by severe infiltration of neutrophils and mononuclear cells in the gastric mucosa [1]. Accumulation and activation of these cells is induced by the local production of cytokines, such as interleukin (IL)-1β. Evidence suggests that *H. pylori* lipopolysaccharide (LPS) mediates release of cytokines from human monocytes [2,3]. The biological effects of these cytokines may result in the recruitment, influx and activation of neutrophils in the gastric mucosa in the event of infection by *H. pylori* [4]. IL-1β is a potent inflammatory cytokine that is released as a component of the host response against bacterial infection. It is expressed primarily by activated macrophages [5].

The heterodimeric cytokine IL-12 plays a key role in host defence by differentiating the cells of T helper 1 (Th1) response. Recently, IL-23 was identified as a member of the IL-12 cytokine family secreted by neutrophils and monocytes. Recent data revealed the involvement of IL-23 in inflammatory procedures of the lower gastrointestinal tract, mainly Crohn's disease [6]. Whether IL-23 contributes to the inflammatory reaction taking place in the gastric mucosa in the event of gastritis is not defined. In the present study, biopsies of gastric mucosa taken from patients with peptic ulcer disease and chronic gastritis were cultured to test the release of IL-23.

Patients and methods

Study group

The study was approved by the Medical and Ethics Committee (6th/11·30·05/26962 and 4th/07·16·06/11573) of General Hospital 'Sismanoglion', Athens, Greece. A total of 111 patients were enrolled; 47 patients with duodenal ulcer, 33 with gastric ulcer and 31 with chronic gastritis without peptic ulcer disease. Clinical and endoscopic data for 72 of these patients have already been published [7].

Informed consent was obtained from all participants. Indications for endoscopy in these patients were abdominal pain or discomfort, epigastric pain with nausea and vomiting and dyspepsia. All endoscopies were performed by the

same endoscopist. Peptic ulcer was defined as a circumscribed break in the mucosa in the duodenum (DU) or in the stomach (GU) with apparent depth covered by an exudate, as described previously [8]. All patients with peptic ulcer disease belonged to the Forrest III score [9]. *H. pylori* infection was defined by the presence of the bacterium both in the histopathological findings of each biopsy and after a gastric biopsy culture with the proper growth medium [10]. Exclusion criteria for the study were: recent upper gastrointestinal (GI) bleeding, gastric carcinoma, diabetes mellitus, liver cirrhosis, acute or chronic renal failure and the ingestion of any anti-microbial or anti-secretory medication for at least 15 days prior to endoscopy.

Study design and interventions

All patients were examined by upper GI endoscopy. All patients were endoscoped; biopsies were collected during each endoscopy. At the time of endoscopy, two biopsy specimens were obtained from adjacent areas of the gastric antrum. When each biopsy specimen was taken, the forceps were opened fully and aimed at right-angles to the gastric lumen to the extent possible to obtain uniformly sized biopsies. Biopsies were obtained from endoscopically intact mucosa distant from focal lesions such as ulcers and erosions. Each biopsy was used for culture.

In brief, gastric antral mucosal biopsy tissues were weighed and cultured on a culture insert over wells containing RPMI-1640 medium with 10% heat-inactivated fetal bovine serum in a 5% CO_2 incubator for 18 h [11]. Biopsies were positioned on the insert with mucosal surfaces uppermost. The first biopsy tissue was left unstimulated and served as control, and the second was stimulated with 10 ng/ml of LPS of *Escherichia coli* O144 : H4. Stimulation with LPS was performed to mimic *in vivo* conditions where gastric and dudodenal mucosa are exposed to LPS of *H. pylori*. At the end of the incubation, the plates were centrifuged for 10 min at 1400 ***g***; supernatants were then collected from the wells and stored at −70°C until assayed for estimation of IL-23 and IL-1β. Results were correlated with histopathological findings.

Estimation of IL-1β and IL-23

The IL-1β was estimated by a developmental enzyme immunoabsorbent assay using capture and detection antibodies and IL-1β standards (R&D Inc., Minneapolis, MN, USA). The lowest limit of detection was 15·6 pg/ml. IL-23 was measured in supernatants with a commercially available enzyme immunoabsorbent assay (eBioscience, San Diego, CA, USA). The lowest limit of detection was 15 pg/ml. All measurements were performed in duplicate and cytokine concentrations were expressed as pg/g of tissue.

Histopathology

Formalin-fixed, paraffin-embedded tissue samples were cut routinely at 3–4 μm and stained with haematoxylin and eosin alcian blue periodic acid Schiff (pH: 2·5) and Giemsa. Immunochemistry was performed for *H. pylori* detection with 1 : 100 dilution of Renoir Red (Biocare Med., Concord, CA, USA).

The presence of gastritis was evaluated in each biopsy sample after separate scoring for each of the following parameters: (i) disease activity as mucosal infiltration by neutrophils; (ii) chronic inflammation expressed as infiltration by monocytes and lymphocytes; (iii) degree of mucosal atrophy; and (iv) intestinal metaplasia. Each parameter was scored from 0 to 3 (0: absent, 1: mild, 2: moderate, 3: marked). In the case of intestinal metaplasia, scores indicated the following findings: 0: absence; 1: complete or type I; 2: incomplete or type II; and 3: incomplete or type III. As a consequence, the total Sydney score of gastritis ranged between 0 and 15, according to previously reported criteria of the updated Sydney System [12]. The extent of gastric inflammation in the antrum, corpus or both was also recorded. The density of *H. pylori* was evaluated semiquantitatively by the same criteria [13]. Specimens were classified by one pathologist who was unaware of the corresponding laboratory findings.

Statistical analysis

Patients of the three groups were divided further into subgroups according to the absence or presence of *H. pylori*. Concentrations of IL-23 and IL-1β were expressed by their mean and standard error for the total number of patients and by their median ±95% confidence intervals or interquartile range when patients were divided further according to the presence or absence of *H. pylori* infection. Updated Sydney classification scores were given by their means (±standard deviation). Comparison between groups was made by Mann–Whitney *U*-test with a correction according to Bonferroni; for qualitative data comparisons were performed by χ^2 test. Comparisons before and after stimulation with LPS was performed by Wilcoxon's paired test. Correlations between IL-23 and IL-1β and the gastritis score or the density of *H. pylori* were performed according to Spearman's rank order. Any *P*-value less than 0·05 was considered significant.

Results

Patients' characteristics are shown in Table 1. No differences were found between the three groups regarding age, sex and intake of non-steroidal anti-inflammatory drugs. All patients, except one, suffering from duodenal ulcer had presence of *H. pylori* on tissue biopsy.

Table 1. Demographic characteristics of patients enrolled in the study.

Parameters	Duodenal ulcer		Gastric ulcer		Chronic gastritis	
Patients (*n*)	47		33		31	
Age (mean ± s.d.)	66·51 ± 9·85		66·27 ± 11·47		64·93 ± 11·54*	
Male/female	29/18		20/13		19/12*	
Non-smoking/smoking	22/25		16/17		14/17*	
History of NSAID use (yes/no)	29/18		20/13		19/12*	
*Helicobacter pylo*ri positive/negative	46/1		21/12		21/10†	
Site of gastric inflammation						
Antrum (no. of patients)	23		16		12*	
Corpus (no. of patients)	13		7		9*	
Disseminated (no of patients)	11		10		10*	
	H. pylori (+)	*H. pylori* (−)	*H. pylori* (+)	*H. pylori* (−)	*H. pylori* (+)	*H. pylori* (−)
Total updated Sydney score (mean ± s.d.)	5·47 ± 2·09	n.a.	6·33 ± 1·87	5·25 ± 2·56	5·09 ± 1·41	4·50 ± 1·70
Degree of neutrophil infiltration (mean ± s.d.)	1·91 ± 0·73	n.a.	2·52 ± 0·60	1·50 ± 0·79‡	2·14 ± 0·79	1·20 ± 0·92‡
Degree of monocyte infiltration (mean ± s.d.)	1·96 ± 0·82	n.a.	1·90 ± 0·77	1·83 ± 0·72	1·86 ± 0·73	1·30 ± 0·82
Degree of lymphocyte infiltration (mean ± s.d.)	0·78 ± 0·89	n.a.	0·76 ± 0·76	1·08 ± 1·16	0·67 ± 0·86	1·00 ± 067
Degree of mucosal atrophy (mean ± s.d.)	0·57 ± 0·83	n.a.	0·76 ± 0·94	0·42 ± 0·67	0·33 ± 0·66	0·70 ± 0·82
Degree of intestinal metaplasia (mean ± s.d.)	0·26 ± 0·55	n.a.	0·38 ± 0·74	0·42 ± 0·67	0·10 ± 0·30	0·30 ± 0·68
Density of *Helicobacter pylo*ri (mean ± s.d.)	1·98 ± 0·91		1·90 ± 0·83		2·10 ± 0·83	

*P = n.s. (non-significant) between the three groups. †$P < 0{\cdot}0001$ between the three groups. ‡$P < 0{\cdot}0001$ compared with the respective *Helicobacter pylori*-positive patients. Updated Sydney scores are given. NSAID, non-steroidal anti-inflammatory drugs; s.d., standard deviation; n.a., not applicable.

The degree of infiltration of the gastric mucosa by neutrophils was greater among *H. pylori*-positive patients compared with *H. pylori*-negative patients. No differences were recorded between them regarding the other histological parameters of gastritis (Table 1).

Concentrations of IL-23 and of IL-1β in supernatants of samples of gastric mucosa taken from patients with duodenal ulcer disease with gastric ulcer disease and with chronic gastritis are shown in Table 2. They were higher among the population of *H. pylori*-positive patients compared with *H. pylori*-negative patients.

Comparisons of concentrations of IL-23 and IL-1β in supernatants before and after stimulation with LPS are shown in Fig. 1. Stimulation with LPS resulted in a significant increase of the production of both IL-23 and IL-1β.

Concentrations of IL-23 in tissue supernatants after stimulation with LPS were correlated positively with the degree of infiltration by monocytes ($P = 0{\cdot}013$, $r^2 = +0{\cdot}498$) and by neutrophils ($P = 0{\cdot}001$, $r_s = +0{\cdot}631$). Similar correlations were found between concentrations of IL-1β after stimulation with LPS with the degree of infiltration by monocytes ($P < 0{\cdot}001$, $r^2 = +0{\cdot}509$) and by neutrophils ($P < 0{\cdot}001$, $r_s = +0{\cdot}553$).

Discussion

Chronic active gastritis is the leading inflammatory disorder of the upper gastrointestinal tract caused by *H. pylori* infection. *H. pylori*-related gastric inflammation may also evolve into peptic ulcer disease [14]. The latter process is

Table 2. Concentrations of interleukin (IL)-23) and IL-1β of supernatants of tissue samples taken from patients with either duodenal or gastric ulcer disease or chronic gastritis.

	Duodenal ulcer		Gastric ulcer		Chronic gastritis	
	H. pylori (+)	*H. pylori* (−)	*H. pylori* (+)	*H. pylori* (−)	*H. pylori* (+)	*H. pylori* (−)
No. of patients	46	1	21	12	21	10
			IL-23 [median (IQR), pg/g of tissue]			
−LPS	223·8 (137·8)	–	1748·3 (332·2)*	138·0 (260·8)	323·3 (60·89)†	204·1 (86·91)
+LPS	355·4 (369·4)	–	289·7 (567·3)*	249·3 (456·1)	534·1 (119·16)‡	377·2 (91·26)
			IL-1β [median (IQR), pg/g of tissue]			
−LPS	723·1 (799·8)	–	617·3 (945·9)†	532·3 (441·1)	782·3 (666·8)†	704·3 (720·4)
+LPS	1593·1 (1663·2)	–	1486·9 (1001·4)*	856·4 (963·2)	1213·9 (1489·7)†	1453·6 (1759·9)

P of comparisons with *Helicobacter pylori*-negative patients: *$P < 0{\cdot}05$, †P = non-significant, ‡$P < 0{\cdot}01$. Patients were divided as either *H. pylori*-positive or *H. pylori*-negative. Lipopolysaccharide (LPS): endotoxin of *Escherichia coli* O144 : H4. IQR, interquartile range.

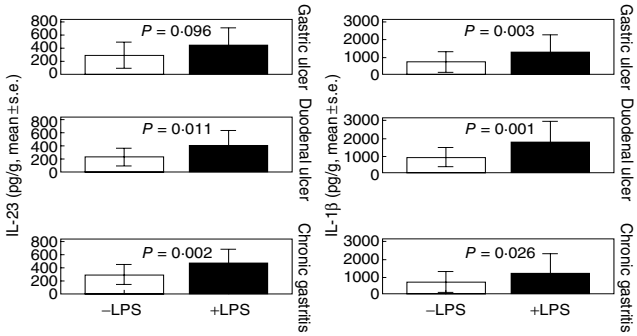

Fig. 1. Concentrations of interleukin (IL)-23 and IL-1β in supernatants of gastric mucosa in patients with duodenal and gastric ulcer, and chronic gastritis before and after stimulation with lipopolysaccharide (LPS). *P*-values refer to comparisons before and after stimulation with LPS.

mediated by various mechanisms implicating several pro-inflammatory cytokines.

Former results of our group revealed that the inflamed gastric mucosa was potent for the release of soluble triggering receptor expressed on myeloid cells (sTREM-1) in peptic ulcer disease. Proinflammatory cytokines such as tumour necrosis factor-α were released from the inflamed gastric mucosa independently from the inflammatory status [7]. Other authors have stated that cell loss and apoptosis of gastric mucous cells was enhanced by *H. pylori* LPS. The latter is less potent compared with *E. coli* LPS for the induction of release of proinflammatory mediators. The low immunological potency of *H. pylori* LPS may contribute to low-grade gastritis [15]. In an attempt to simulate the latter process, cultured biopsies were stimulated with LPS. The applied LPS derived from *E. coli* O144 : H4 acts as a Toll-like receptor-4 agonist. It was used for this study because of the lack of commercially available of *H. pylori*. It should be emphasized that this may also be a limitation of our study, as LPS of *H. pylori* has also been described to behave in a different manner [16].

Results revealed that the inflamed gastric mucosa of *H. pylori*-positive patients could secrete IL-23 (Fig. 1). Gastric mucosa of patients with both duodenal and gastric ulcers was equally potent for secretion of IL-23 compared with patients with chronic active gastritis with no signs of peptic ulcer disease. The release of IL-23 was greater by *H. pylori*-infected gastric mucosa than by gastric mucosa not infected by *H. pylori* mainly for patients with chronic gastritis and only after stimulation with LPS. Similar findings have been published elsewhere [17,18].

Release of IL-23 after stimulation of gastric mucosa of patients with gastric ulcer and *H. pylori* infection was reduced. This probably implies a different effect of *E. coli* LPS and *H. pylori* on the gastric mucosa regarding the release of IL-23 in the event of gastric ulcer.

Similar kinetics was also observed for IL-1β. IL-1β was increased in strict correlation with the degree of mucosal inflammation independently from the underlying pathogenetic status.

The release of IL-23 in supernatants of gastric mucosa in peptic ulcer disease and chronic gastritis creates the hypothesis that IL-23 is a cytokine implicated in the pathogenesis of chronic gastric inflammation. Both IL-23 and IL-1β were correlated with the degree of acute and chronic inflammation. IL-23 belongs to the IL-12 superfamily, which is implicated in the Th1 immune response. On that basis, it might be proposed that IL-23 participated in the orchestration of chronic inflammation of the gastric mucosa.

The present study has reported for the first time in the literature two separate findings for the capacity of inflamed gastric mucosa for the release of proinflammatory cytokines. First, gastric mucosa secretes IL-23 at a rate dependent on the degree of infiltration by inflammatory cells; and secondly, the kinetics of release of IL-23 followed those of IL-1β. Whether IL-23 is an independent mediator in the pathogenesis of peptic ulcer disease or not cannot be excluded with safety from the presented findings. It is anticipated that IL-23 participated in the process of inflammation irrespective of the presence or not of ulcer. Further investigation is necessary to elucidate fully the exact role of IL-23 in the pathogenesis of chronic gastritis.

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Alimentary Tract

Endothelial nitric oxide synthase (eNOS) is not upregulated in gastric mucosa of *Helicobacter pylori* (*H. pylori*)-positive patients with type 2 diabetes mellitus

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Abstract

Aim. To evaluate the expression of eNOS and CD34 in gastric mucosa of *Helicobacter pylori* (*H. pylori*) positive diabetic patients, in correlation with glycaemic control and diabetic autonomic neuropathy (DAN).

Methods. We prospectively studied 49 diabetic type 2 patients (29 women, mean age 65.32 ± 8.56 years) and 30 control subjects (15 women, mean age 58.47 ± 12.40) that underwent endoscopy. Biopsies from the body and antrum were evaluated for *H. pylori*-gastritis, eNOS and angiogenic marker CD34 expression. Statistical analysis in correlation with mean glycosylated haemoglobin (HbA1c) of the last 3 years, and DAN was performed.

Results. The two groups were matched for age ($p = 0.144$), sex ($p = 0.335$), *H. pylori*-infection ($p = 0.617$) and degree of gastritis ($p = 0.78$). eNOS and CD34 attenuated expression correlated with diabetes mellitus (DM) in the corpus ($p = 0.009$ and 0.02, respectively). eNOS and CD34 expression was upregulated in *H. pylori*-positive controls but not in *H. pylori*-positive diabetic patients ($p = 0.010$ and 0.007 for the corpus and $p = 0.036$ and 0.047 for the antrum, respectively). eNOS expression correlated with good glycaemic control (GGC) in the gastric corpus ($p < 0.001$) and antrum ($p = 0.0037$) and with absence of DAN ($p = 0.009$ and 0.036, respectively for the corpus and antrum).

Conclusion. Chronic glycaemic control affects eNOS expression and angiogenesis in the gastric mucosa of patients with type 2 DM. eNOS expression is not upregulated in *H. pylori*-positive diabetic patients.
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Keywords: eNOS; Nitric oxide; Diabetes; Gastric defence; Submucosal blood flow; Angiogenesis; *H. pylori*

1. Introduction

Nitric oxide (NO) plays a multifaceted role in mucosal integrity. The several functions of NO and the double-edged role played by NO in most of them provide a great complexity to the NO action. The three enzymatic sources of NO, neuronal NO-synthase (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) have been recognized in the gastrointestinal tract. The protective properties of the NO derived from constitutive NO synthases (eNOS and nNOS) have already been well established. Less clear is the role assigned to iNOS [1]. Constitutively produced NO is believed to be an important component of mucosal defence mechanisms, primarily because it increases mucosal blood flow. NO is an

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important modulator of mucosal repair, probably because of its ability to enhance collagen deposition by fibroblasts and to stimulate angiogenesis [2]. NO is implicated in maintaining the gastric epithelium intercellular barrier integrity and in regulating secretory processes in the gastric mucosa; it protects the gastric mucosa by acting as a vasodilator that, similarly to calcitonin gene related peptide (CGRP), increases gastric mucosal blood flow (GMBF) [3]. It also increases secretion of mucus, and may down-regulate gastric acid secretion. In particular, NO interacts with neuropeptides and prostaglandins to maintain mucosal integrity in basal conditions [4]. Evidence suggests that this is a dynamic interaction, in which when an element is suppressed others assume its function.

In diabetes mellitus (DM), evidence for a reduced NO bioactivity has been found [5] either due to a decreased NO production, a reduced availability of the substrate L-arginine for the NO producing enzyme, or an increased destruction of NO by reactive oxygen metabolites (ROMs). It has been suggested that an altered action of the vascular endothelial cells might contribute to the development of diabetic vascular complications [6].

Gastrointestinal disorders occur in as many as 75% of diabetic patients. Studies in streptozotocin (STZ)-induced diabetic rats, an accepted model of insulin-dependent diabetes [7], have demonstrated increased gastric mucosa susceptibility [7–12] and impaired gastric lesions healing implicating multiple possible mechanisms [7–16]. It is possible that the impaired GMBF response in diabetic rats is due to decrease release and/or production of endogenous NO, in addition to a dysfunction in capsacain-sensitive sensory neurons [7,17]. So far, data from experimental studies concerning the NOS activity in gastric mucosa of diabetic animals have been contradictory [10,18].

Recently, we reported that eNOS expression is upregulated in gastric mucosa of *Helicobacter pylori* (*H. pylori*)-positive patients, along with CD34 overexpression, a well-known angiogenesis marker. However, eNOS was not correlated with the degree of gastric inflammation suggesting that eNOS triggering happens early in the inflammation cascade and has an important role in preserving homeostasis in the interaction between the endothelium and inflammatory cells [19].

In this study we evaluated the impact of *H. pylori* infection on eNOS expression and angiogenesis, estimated by the microvessel density marker CD34, in the gastric mucosa of diabetic patients with possible correlation to glycaemic control and diabetic complications.

2. Materials and methods

2.1. Subjects' selection

We prospectively studied 49 non-smoking patients with type 2 DM and 30 non-smoking, non-diabetic controls referred to our endoscopy unit because of dyspepsia (epigastric pain, bloating, abdominal discomfort, esophageal and/or epigastric burning).

Diabetic patients with at least 5-year duration of the disease treated with insulin and followed in the diabetes outpatient clinic for the previous 3 years (2–4 visits per year) were included in the study group. Control subjects were considered eligible when no history of DM was reported and fasting blood glucose levels were <110 mg/dL. All control subjects that were eventually eligible for the study underwent an oral glucose tolerance test.

Before endoscopy all diabetic patients with blood glucose measured levels >200 mg/dL were excluded from the study. All subjects (patients and controls) with gastric and/or duodenal lesions diagnosed by endoscopy were also excluded from the study. None of the subjects had been drinking alcohol or taking corticosteroids, antibiotics, non-steroidal anti-inflammatory or anti-secretory drugs for at least one month before the endoscopy. Subjects with a history of gastric and/or duodenal ulcer, as well as those who received eradication treatment or gastric surgery were excluded from the study. Additional exclusion criteria included hypertension, renal insufficiency, dyslipidaemia under treatment, chronic obstructive pulmonary disease, heart failure, cirrhosis and inflammatory bowel disease.

All participants gave informed consent to enter the study. The procedures followed in this study were in agreement with the ethical standards of the committee on human experimentation of the institution in accordance with the Declaration of Helsinki.

2.2. Subgroups

Before endoscopy, all patients, after an 8–12 h fasting, were measured for blood glucose levels. Patients' glycaemic control was rated on the basis of mean glycated Hb (HbA_{1c}) (%) values of the last 3 years (at least 6 values). Patients were considered to have good glycaemic control with a mean $HbA_{1c} < 7.0\%$ [GGC subgroup] and poor glycaemic control with a mean $HbA_{1c} \geq 7.0\%$ [PGC subgroup].

All patients with DM were referred for cardiologic examination. Patients were considered to suffer from diabetic autonomic neuropathy (DAN) when diagnosis of cardiologic autonomic neuropathy (CAN) as defined by abnormal CAN tests [20] was established in their history [DAN group]. Diabetic patients without CAN were included in the non-DAN group.

2.3. Endoscopy and tissue sampling

Subjects reported at 9 AM after a 12-h fasting. Intravenous sedation with midazolam (2–5 mg) was given, and standard upper gastrointestinal endoscopy was performed with a forward viewing videoscope (Olympus GIF-160; Opto-Electronics Co. Ltd., Tokyo, Japan) to identify evidence of macroscopic abnormalities; in all participants, the gastric and the duodenal mucosa appeared to be without obvious macro-

scopic abnormalities. Simultaneously, two biopsy specimens were obtained with pinch biopsy forceps (Olympus FB 24K-1) from the antral region within 2 cm of the pyloric ring and three from the corpus (two from the greater and one from the lesser curvature).

H. pylori detection methods were biopsy urease test and histopathology. All specimens were stained with haematoxylin and eosin. For detection of *H. pylori* organisms, Crezyl fast violet and/or Giemsa stains were preferred. Moreover, intestinal metaplasia was evaluated with Alcian blue stain. Gastritis was scored according to a modified Sydney classification [21]. Two experienced pathologists (V.E. and V.T.) blinded to clinical information assessed all specimens separately and then reviewed together histological grading, eNOS and CD34 expression.

2.4. eNOS immunolocalisation

Immunohistochemistry was performed as described previously [22]. In brief, 4-μm-thick serial sections were cut from paraffin blocks, mounted on acid cleaned glass slides, and heated at 55 °C for 60 min. Slides were dewaxed and rehydrated; then the endogenous peroxidase activity was inhibited by incubation with 0.5% H_2O_2 in methanol (10 min at room temperature). To reduce non-specific background staining, slides were incubated with RTU Normal Horse serum (10 min at room temperature). To enhance immunostaining, sections were treated with an antigen retrieval solution (10 mM citric acid monohydrate, pH 6.0, adjusted with 2N NaOH) and heated three times in a microwave oven at high power for 5 min. Then, sections were washed in buffer solution TBS pH 7.6 (buffer 50 mM Tris, 0.15 M NaCl, pH 7.6 TBS) for 5 minutes and incubated in the primary antibody, a 1:40 dilution of mouse monoclonal antibody against human NOS-3 (NCL-NOS-3, Novocastra Laboratories Ltd, United Kingdom). Then, the samples were washed with PBS for 10 min and incubated with a biotinylated secondary antibody. Sections were rinsed with PBS and incubated with streptavidin–biotin–peroxidase complex (Dako Corp.) for 20 min at room temperature. Sections were developed with chromogen DAB and then counterstained with haematoxylin, dehydrated, and mounted.

The degree of immunopositivity for eNOS was evaluated semi-quantitatively. In random fields from representative areas of the gastric biopsies, the immunoreactive cells were roughly assessed and expressed as a percentage. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm (NOS III, eNOS). The intensity of immunopositivity was graded as 0 (<5% cell with positive staining), 1 (5–30%), 2 (30–60%), or 3 (>60%), and expressed as mean ± standard deviation (S.D.). Two independent approaches were used to confirm antibody specificity: (1) serial dilution of the primary antibody until the immunohistochemical signal disappeared, and (2) the use of non-immune mouse IgG instead of primary antibodies which failed to reveal relevant staining.

2.5. Neovascularisation analysis

Immunohistochemical staining of CD34+ endothelial cells was performed to analyse the degree of angiogenesis in the gastric mucosa of gastritis patients. For CD34 staining, tissue sections were processed and stained with a 1:50 dilution of a mouse monoclonal against human CD34 antibody (NCL-END, Novocastra Laboratories Ltd., United Kingdom) and peroxidase-conjugated anti-mouse IgG and then counterstained with haematoxylin. The slides were mounted with the sections and examined using a bright-field microscope. A positive reaction was indicated by a reddish brown precipitate in the cytoplasm.

The number of blood vessels per gastric mucosa specimen was calculated by counting the CD34-positive vessels, including mono-endothelial vessels, in at least five different fields of representative areas, at 100× magnification (0.25 mm × 0.35 mm). Leica IM50—Image Manager Software applied to a light microscope was used for computer analysis of each sample. Vascularisation of gastric mucosa was expressed as mean number of capillaries per biopsy (mean vascular density, MVD). The MVD levels were divided into four groups: (0) 0–20 vessels; (1) 20–40 vessels; (3) 40–60 vessels; (4) >60 vessels.

2.6. Statistical analysis

The significance of differences was evaluated using the Pearson's chi-square test (SPSS Statistical Package, Chicago IL, USA). All p values were two tailed. Significance was set at $p < 0.05$.

3. Results

After detailed interview for exclusion criteria, 136 patients were eligible for inclusion to the study at the time of referral for endoscopy. Thirty-five patients were excluded because of gastric or duodenal lesions observed during endoscopy (gastric cancer in 1 patient; duodenal ulcers in 21 patients; duodenal erosions in 9 patients; and gastric erosions in 9 patients). Sixteen diabetic patients presented with blood glucose measured levels >200 mg/dL before endoscopy and were also excluded from the study, whereas 5 patients did not present for endoscopy. Therefore, 49 patients with at least 5-year duration of DM type 2 (29 women, mean age 65.32 ± 8.56 years) and 30 non-diabetic dyspeptic patients (15 women, mean age 58.47 ± 12.4 years) were finally included into the study. The two groups (diabetic patients vs. controls) were matched for age ($p = 0.144$), sex ($p = 0.335$), *H. pylori* infection incidence ($p = 0.617$) and degree of gastritis (Table 1), according to the modified Sydney classification [21]. Twenty four patients presented with mean $HbA_{1c} < 7\%$ (mean $HbA_{1c} = 5.75 \pm 0.82$, GGC subgroup) and 25 presented with mean $HbA_{1c} \geq 7\%$ (mean $HbA_{1c} = 8.8 \pm 1.8$, PGC subgroup). Twenty-one diabetic patients with mean

Table 1
Histologic findings in diabetic patients and controls

	Gastric corpus			Gastric antrum		
	Controls, $N=30$ (%)	Patients, $N=49$ (%)	p	Controls, $N=30$ (%)	Patients, $N=49$ (%)	p
Gastritis						
No inflammation	11 (33.34)	10 (20.4)	0.431	12 (40.0)	3 (6.1)	0.041
Low	6 (18.19)	20 (40.8)		6 (20.0)	13 (26.5)	
Moderate	10 (30.3)	15 (30.6)		9 (30.0)	26 (53.06)	
Severe	3 (10.0)	4 (8.1)		3 (10.0)	7 (14.3)	
Gastritis activity						
No activity	24 (80.0)	39 (79.59)	0.91	19 (63.3)	32 (65.3)	0.705
Low	3 (10.0)	6 (15.4)		6 (18.19)	7 (14.3)	
Moderate	2 (6.6)	2 (5.1)		2 (6.6)	6 (12.2)	
Severe	1 (3.3)	2 (5.1)		3 (10.0)	4 (8.2)	
Atrophic gastritis						
Without findings	26 (86.6)	45 (91.8)	0.592	20 (66.6)	35 (71.42)	0.199
Low	3 (10.0)	2 (4.08)		7 (23.3)	4 (8.1)	
Moderate	1 (3.3)	2 (4.08)		3 (10.0)	10 (20.4)	
Severe	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Intestinal metaplasia						
Without findings	30 (100.0)	48 (97.9)	0.61	28 (93.3)	47 (95.9)	0.65
Low	0 (0.0)	1 (2.1)		2 (6.6)	2 (4.08)	
Moderate	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Severe	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	

$HbA_{1c} \geq 7\%$ presented DAN (mean $HbA_{1c} = 8.7 \pm 1.04$) (Table 2).

No significant differences were observed in the distribution of gastritis between GGC and PGC subgroups (Table 3). Chronic gastritis and gastritis activity distributions were similar between diabetic patients with and without DAN ($p = 0.341$ and 0.149, respectively in the gastric corpus and $p = 0.859$ and 0.293, respectively in the antrum).

Three main distribution patterns of eNOS immunoreactivity were observed as previously described [23]: along the basolateral surface of mucous cells, in the endothelial cells of mucosal vessels and in cells within gastric glands, located near the basal membrane. Intensity of eNOS and CD34 expression in the corpus and antrum were significantly correlated ($p < 0.001$). CD34 immunohistochemical staining was not accomplished in 1 control patient in the gastric antrum and in 1 control patient in the corpus. eNOS expression significantly correlated with *H. pylori* infection in the mucosa of the corpus and antrum ($p = 0.013$ and 0.020, respectively). Gastric inflammation and activity were not correlated with eNOS expression ($p = 0.848$ and 0.871, respectively in the corpus and $p = 0.565$ and 0.793, respectively in the antrum). eNOS expression was correlated with GGC in the gastric corpus ($p < 0.001$) and antrum ($p = 0.0037$) (Table 4). Similarly, eNOS expression in the gastric corpus and antrum was correlated with absence of DAN ($p = 0.009$ and 0.036).

In all patients, CD34 expression was not correlated with gastric inflammation and activity in the gastric mucosa of the corpus ($p = 0.358$ and 0.520, respectively). In the gastric antrum, CD34 expression was significantly correlated with gastric inflammation ($p = 0.047$) and with a trend to be correlated with activity ($p = 0.050$). CD34 expression in the gastric corpus and antrum was correlated with absence of DAN ($p = 0.001$ and 0.002). Patients with *H. pylori* infection showed higher expression of CD34 positive blood vessels in the gastric mucosa layer of the antrum than those without *H. pylori* infection ($p = 0.048$).

Distribution of eNOS and CD34 expression in the two groups are shown in Table 5. eNOS and CD34 attenuated expression were correlated with DM in the gastric corpus ($p = 0.009$ and 0.02, respectively) but not in the antrum ($p = 0.37$ and 0.16). Subanalysis regarding *H. pylori* infection subgroups demonstrated eNOS (Fig. 1) and CD34 (Fig. 2) upregulated expression in *H. pylori* positive controls than in *H. pylori* positive diabetic patients (Figs. 3 and 4) in the corpus ant the antrum (Tables 6 and 7).

Table 2
Clinical characteristics of patients with good glycaemic control (GGC) and poor glycaemic control (PGC), and diabetic autonomic neuropathy (DAN)

	Patients with GGC ($N=24$)	Patients with PGC ($N=25$)	Patients with DAN ($N=21$)
Mean age ± S.D.	66.07 ± 8.8	65.44 ± 6.63	64.5 ± 7.4
Mean duration of disease ± S.D.	14.72 ± 7.85	16.4 ± 7.63	17.91 ± 6.06
Mean HbA_1c (%) ± S.D.	5.75 ± 0.82	8.81 ± 1.8	8.71 ± 1.04
H. pylori infection	10 (41.6%)	10 (40%)	8 (38.09%)

Table 3
Histologic findings distribution between diabetic patients with good glycaemic control (GGC) and poor glycaemic control (PGC)

	Gastric corpus			Gastric antrum		
	GGC group, $N=24$ (%)	PGC group, $N=25$ (%)	p	GGC group, $N=24$ (%)	PGC group, $N=25$ (%)	p
Gastritis						
No inflammation	4 (16.6)	6 (24.0)	0.771	1 (4.1)	1(6.7)	0.822
Low	11 (45.83)	9 (36.0)		7 (29.16)	6(24.0)	
Moderate	7 (29.1)	8 (32.0)		13 (54.1)	13(52.0)	
Severe	2 (8.3)	2 (8.0)		3 (12.5)	4(16.0)	
Gastritis activity						
No activity	19 (79.1)	20 (80.0)	0.139	17 (70.8)	15(60.0)	0.797
Low	4 (16.6)	2 (8.0)		3 (12.5)	4(16.0)	
Moderate	1 (4.16)	1 (4.0)		2 (8.3)	4(16.0)	
Severe	0 (0.0)	2 (8.0)		2 (8.3)	2(8.0)	
Atrophic gastritis						
Without findings	22 (91.6)	23 (92.0)	0.489	16 (66.6)	19(76.0)	0.322
Low	2 (8.3)	0 (0.0)		3 (12.5)	1(4.0)	
Moderate	0 (0.0)	2 (8.0)		5 (20.8)	5(20.0)	
Severe	0 (0.0)	0 (0.0)		0 (0.0)	0(0.0)	
Intestinal metaplasia						
Without findings	24 (100.0)	23 (92.0)	0.352	22 (91.6)	15(100.0)	0.445
Low	0 (0.0)	1 (4.0)		2 (8.3)	0(0.0)	
Moderate	0 (0.0)	1 (4.0)		0 (0.0)	0(0.0)	
Severe	0 (0.0)	0 (0.0)		0 (0.0)	0(0.0)	

Table 4
eNOS and CD34 expression distribution in gastric mucosa diabetic patients with good glycaemic control (GGC) and poor glycaemic control (PGC)

eNOS	Gastric corpus			Gastric antrum		
	GGC group $N=24$ (%)	PGC group $N=25$ (%)	p	GGC group $N=24$ (%)	PGC group $N=25$ (%)	p
0 (<5%)	0 (0.0)	3 (12.0)	<0.001	0 (0.0)	0 (0.0)	0.0037
1 (5–30%)	4 (16.6)	21 (84)		0 (0.0)	9 (36.0)	
2 (30–60%)	16 (66.6)	1 (4.0)		10 (41.6)	15 (60.0)	
3 (>60%)	4 (16.6)	0 (0.0)		14 (58.33)	1 (4.7)	

CD34	Gastric corpus			Gastric antrum		
	GGC group, $N=24$ (%)	PGC group, $N=24$ (%)	p	GGC group, $N=24$ (%)	PGC group, $N=24$ (%)	p
0 (0–20)	5 (20.8)	18 (75.0)	0.0022	0 (0.0)	10(41.6)	0.0019
1 (20–40)	16 (66.6)	6 (25.0)		14 (58.33)	14(58.33)	
2 (40–60)	3 (12.5)	0 (0.0)		10 (41.6)	0(0.0)	
3 (>60)	0 (0.0)	0 (0.0)		0 (0.0)	0(0.0)	

Table 5
eNOS and CD34 expression in gastric mucosa of corpus and antrum in diabetic patients and controls

eNOS	Gastric corpus			Gastric antrum		
	Controls, $N=29$ (%)	Patients, $N=49$ (%)	p	Controls, $N=29$ (%)	Patients, $N=49$ (%)	p
0 (<5%)	0 (0.0)	3 (6.122)	0.009	0 (0.0)	0 (0.0)	0.37
1 (5–30%)	3 (10.34)	25 (51.02)		2 (6.9)	9 (18.36)	
2 (30–60%)	14 (48.2)	17 (34.69)		14 (48.2)	25 (51.02)	
3 (>60%)	12 (41.4)	4 (8.16)		13 (44.8)	15 (30.06)	

CD34	Gastric corpus			Gastric antrum		
	Controls, $N=28$ (%)	Patients, $N=48$ (%)	p	Controls, $N=28$ (%)	Patients, $N=48$ (%)	p
0 (0–20)	6 (21.4)	23 (47.91)	0.02	12 (42.8)	10 (20.83)	0.16
1 (20–40)	11 (39.28)	23 (47.91)		9 (32.14)	28 (58.33)	
2 (40–60)	11 (39.28)	3 (6.25)		7 (25.0)	10 (20.83)	
3 (>60)	0 (0.0)	0 (0.0)		0 (0.0)	0(0.0)	

Table 6
eNOS and CD34 distribution in gastric corpus mucosa of *Helicobacter pylori* (*H. pylori*) positive and negative diabetic patients and controls

eNOS	*H. pylori* positive			*H. pylori* negative		
	Controls, $N = 12$ (%)	Patients, $N = 20$ (%)	p	Controls, $N = 17$ (%)	Patients, $N = 29$ (%)	p
0 (<5%)	0 (0.0)	2 (10.0)	0.010	0 (0.0)	1 (3.4)	0.627
1 (5–30%)	0 (0.0)	10 (50.0)		3 (17.6)	15 (51.72)	
2 (30–60%)	2 (16.6)	7 (35.0)		12 (70.6)	10 (34.5)	
3 (>60%)	10 (83.3)	1 (5.0)		2 (11.76)	3 (10.34)	
CD34	*H. pylori* positive			*H. pylori* negative		
	Controls, $N = 12$ (%)	Patients $N = 20$ (%)	p	Controls $N = 17$ (%)	Patients $N = 29$ (%)	p
0 (0–20)	0 (0.0)	8 (40.0)	0.007	6 (35.29)	15 (51.72)	0.089
1 (20–40)	5 (41.6)	12 (60.0)		6 (35.29)	11 (37.9)	
2 (40–60)	7 (58.3)	0 (0.0)		5 (29.4)	3 (10.34)	
3 (>60)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	

Table 7
eNOS and CD34 distribution in gastric antrum mucosa of *Helicobacter pylori* (*H. pylori*) positive and negative diabetic patients and controls

eNOS	*H. pylori* positive			*H. pylori* negative		
	Controls, $N = 12$ (%)	Patients, $N = 20$ (%)	p	Controls, $N = 17$ (%)	Patients, $N = 29$ (%)	p
0 (<5%)	0 (0.0)	0 (0.0)	0.036	0 (0.0)	0 (0.0)	0.11
1 (5–30%)	0 (0.0)	2 (10.0)		2 (11.7)	7 (24.13)	
2 (30–60%)	4 (25.0)	12 (60.0)		10 (58.8)	13 (44.8)	
3 (>60%)	8 (75.0)	6 (30.0)		5 (29.4)	9 (31.03)	
CD34	*H. pylori* positive			*H. pylori* negative		
	Controls, $N = 12$ (%)	Patients, $N = 20$ (%)	p	Controls, $N = 17$ (%)	Patients, $N = 29$ (%)	p
0 (0–20)	2 (16.6)	7 (35.0)	0.047	10 (58.8)	3 (10.34)	0.831
1 (20–40)	3 (25.0)	12 (60.0)		6 (35.3)	16 (55.1)	
2 (40–60)	7 (58.3)	1 (5.0)		1 (5.9)	8 (27.6)	
3 (>60)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	

4. Discussion

Gastrointestinal disorders, including delayed gastric emptying, diarrhea, constipation, and abdominal pain occur in as many as 75% of diabetic patients; however, whether diabetic patients are principally more susceptible to gastric mucosal injury remains controversial. Recently, Lazaraki et al. [19] reported that *H. pylori* infection upregulates eNOS, and induces angiogenesis, contributing to *H. pylori*-associated pathophysiology in gastric mucosa of non-smoking dyspeptic subjects. Extending these findings, the aim of the present series was to investigate the impact of *H. pylori* infection

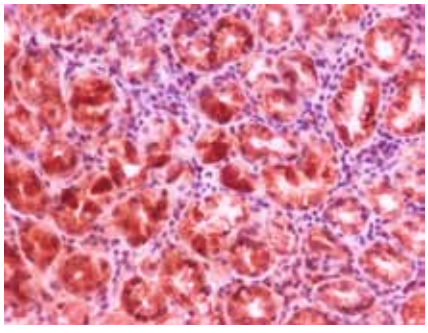

Fig. 1. eNOS overexpression in *H. pylori* positive control subject 400×.

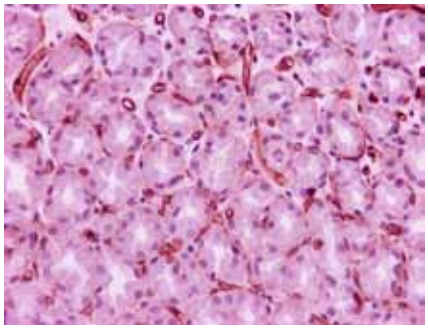

Fig. 2. CD34 overexpression in *H. pylori* positive control subject 400×.

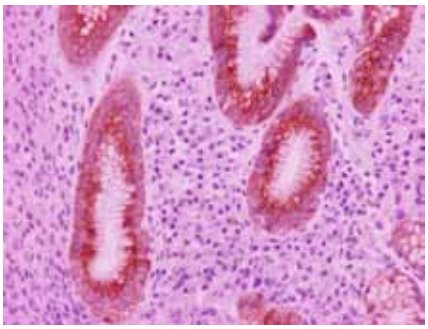

Fig. 3. eNOS attenuated expression in *H. pylori* positive diabetic patient.

and glycaemic control on eNOS expression and angiogenesis induction, estimated by the microvessel density marker CD34, in human gastric mucosa of patients with type 2 DM. To the best of our knowledge, this impact on humans has not been evaluated.

We found that eNOS expression was not correlated with inflammation in diabetic patients and controls. eNOS was distributed in all anatomical regions (cardia, corpus and antrum) as described previously [19,23]: along the basolateral surface of mucous cells, creating a distinct web of immunoreactivity at the surface and in the pit region of the gastric mucosa. eNOS expression was also detected in endothelial cells of mucosal vessels and in cells near the gastric glands of the body and the antrum. Until recently, eNOS has been regarded as a 'static' enzyme that produces a constant amount of NO in both physiological and pathological conditions. This concept has now been abolished and it is clear that it is a far more complicated enzyme than originally anticipated [24]. During the inflammatory process, eNOS upregulation is an early event, being the result to several stimuli including shear stress, vascular endothelial growth factor (VEGF) and autacoids generated locally by tissue injury. NO blocks leukocyte-endothelial adhesion by preventing the synthesis

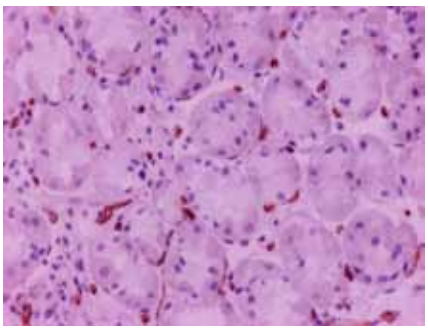

Fig. 4. CD34 attenuated expression in *H. pylori* positive diabetic patient.

of endothelial cell adhesion molecules such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and mucosal addressin cell adhesion molecule-1 resulting in inhibition of leukocyte infiltration at the sites of inflammation. It is evident that eNOS-derived NO has a role in some of the key features of inflammation, such as cell rolling, adhesion and extravasation, as well as in modulating vascular permeability and angiogenesis in vivo [24]. However, in the present series, eNOS was not correlated to the degree of gastric inflammation suggesting that eNOS triggering happens early in the inflammation cascade and has an important role in preserving homeostasis in the interaction between the endothelium and inflammatory cells [24]. Similar findings we also observed in dyspeptic patients [19].

Importantly, we demonstrated that eNOS is poorly expressed in human gastric mucosa of diabetic patients and related to poor glycaemic control, thereby implicated in the pathophysiology of this disease. Specifically, in DM, the bioavailability and activity of endothelium derived NO is reduced due to advanced glycation products (AGEs), the modified proteins or lipids that become nonenzymatically glycated and oxidized after contact with aldose sugars. Key factors crucial to the formation of AGEs include the rate of turnover of proteins for glycoxidation, the degree of hyperglycaemia, and the extent of oxidant stress in the environment [25]. The levels of serum AGEs in patients with type 2 DM are inversely related to the degree of endothelium-dependent and endothelium-independent vasodilation. Several mechanisms by which AGEs reduce or block NO activity have been proposed, including increased rate of eNOS mRNA degradation and reduced eNOS activity [26] and impaired NO production via the binding of N^{e}-(carboxymethyl) lysine residues to endothelial AGE receptors, causing a reduction in phosphorylation of serine residues in eNOS, resulting in deactivation of the enzyme [27]. In this respect, decreased eNOS expression was also demonstrated in endothelial cells of diabetic patients [28].

Constitutively produced NO seems to play a critical protective role in modulating several components of gastric mucosal defence. Recently, Guan et al. [17] have shown that GLP-2R, a peptide implicated in the control of local blood flow and epithelial integrity, motility and secretion, is co-localized with eNOS in submucosal neurons, which function as primary vasomotor effectors to induce vasodilatation of submucosal arterioles. Thus, it is possible that GLP-2R-mediated NO release from submucosal neurons acts as a nonadrenergic, noncholinergic neurotransmitter, which diffuses locally to the vascular smooth muscle to activate cyclic guanosine monophosphate synthesis and stimulate vascular relaxation and arteriole dilation [29]. Several studies have shown a deficiency in the mechanisms underlying the NO-mediated vascular response in diabetic rats [7–12]. Relative data [8], and in particular decreased NOS activity [10], suggest that long-lasting hyperglycaemia may impair the NO-mediated regulation of gastric blood flow. Therefore, we can speculate that decreased endogenous NO synthesis

due to eNOS attenuated expression in our diabetic patients possibly results in gastric mucosa increased susceptibility via defective vasorelaxation and impaired hyperemic response.

Another important finding of this study was that eNOS expression was not upregulated in the corpus and the antrum of *H. pylori* positive diabetic patients. However, in the controls participants of this series and also in a previous work [19], we have demonstrated that eNOS is overexpressed in the *H. pylori*-infected gastric mucosa of non-diabetic dyspeptic study subjects, irrespectively of the degree of gastric inflammation. *H. pylori* gastric infection induces an active immune response including stimulation of iNOS and eNOS expression. Chronic *H. pylori* infection induces iNOS expression, subsequent DNA damage and enhanced antiapoptosis signal transduction sequence [30]. As upregulation of eNOS expression is an early event in the inflammatory process, induced by a variety of stimuli including VEGF, it is possible that the endogenous produced by eNOS NO contributes in ulcer healing and gastric mucosa restitution by inducing angiogenesis in inflammatory tissue [1,31]. Data from patients receiving aspirin, a well-known ulcerogenic agent, show an increase in expression of eNOS in gastric mucosa; this might contribute to reparation of initial lesions, and be responsible for gastric adaptation to chronic aspirin intake in humans [32]. Therefore, the observed eNOS upregulation in *H. pylori*-infected gastric mucosa in our control subjects could also represent a compensatory protective mechanism that seems unable to be achieved in patients with DM.

In this trial, the intensity of eNOS and the microvessel density marker CD34 expression were significantly correlated in diabetic patients and control participants. Relative data indicate that NO has angiogenic properties while eNOS inhibitors impair angiogenesis in granulation tissue during gastric ulcer healing [33]. Following stimulation with the proangiogenic factor VEGF, endothelial cells recruit eNOS and Akt to adjacent regions of the same domain of HSP90, facilitating the phosphorylation of eNOS. Thus, a regulatory cascade is involved in the shift of eNOS to a higher level of activation [24].

During the healing process of acute gastric mucosal necrosis such as erosions or ulcers, the growth of new microvessels is promoted by angiogenic growth factors such as basic fibroblast growth factor (bFGF), VEGF, platelet-derived growth factor, and angiopoietin [33]. In this study, decreased angiogenesis as indicated by CD34 expression was demonstrated in the gastric corpus mucosa of diabetic patients correlated to poor glycaemic control and presence of DAN, thereby suggesting defective aforementioned reconstruction processes. Our data are in agreement with data form experimental studies suggesting attenuation of angiogenesis in the gastric mucosa of diabetic animals due to suppression of bFGF production [13] and dysfunction of capsaicin-sensitive afferent neurons [7].

Decreased eNOS expression was also correlated to the presence of DAN in our diabetic patients. This finding can be partly explained by the evidence that diabetic complications occur in patients with longstanding poor glycaemic control. Traditionally, diabetic gastropathy was considered a part of diabetic neuropathy occurring in up to 40% of patients in a 10-year period [34]. The cause of gastropathy in DM is considered to be multifactorial. Potential causes include vagal nerve dysfunction, sympathetic nerve dysfunction, damage to the enteric nerve system and hyperglycaemia, which can itself damage gastrointestinal function [35]. Moreover, lower gastric interstitial cells of Cajal (ICC), nNOS, and substance P densities in patients with DM might be connected with the pathogenesis of diabetic gastroparesis [36]. ICCs possess receptors for neurotransmitters and such circulating hormones as NO and substance P. This endogenous NO is produced not only by nNOS but also by eNOS, which is not only localized to endothelial cells but also in brain and peripheral neurons [including enteric neurons [37] and sensory neurons [38]]. When the relaxation response of NO and its mediation in diabetic rats were studied [39], the number of NOS-immunoreactive cells in the gastric myenteric plexus and NOS activity were significantly reduced in diabetic BB/W rats, suggesting that NOS synthesis is impaired in DM. These results show that gastric relaxation in diabetic patients is hampered mainly by impaired NOS expression in the gastric myenteric plexus [40]. Although eNOS expression in submucosal neurons was not in the objectives of the present study, and therefore not demonstrated, eNOS decreased expression in the gastric mucosa is reported for the first time to be correlated with the presence of clinical neuropathy in diabetic patients, thereby indicating a possible implication of impaired eNOS expression in the mechanism of diabetic gastropathy.

A limitation of our study is the rather small number of participants possibly due to strict selection and exclusion of patients with endoscopic lesions. Since CD34 is a known neovascularisation marker, we selected patients without obvious endoscopic lesions because it would be inappropriate to compare, for example, specimens with increased angiogenesis during the healing process with normal gastric mucosa. Although the correlation between microscopic and endoscopic abnormalities is poor, in this study, histology findings were comparable between the two groups.

In conclusion, our study demonstrated for the first time that eNOS expression is attenuated in gastric corpus mucosa of *H. pylori* positive diabetic patients in comparison with *H. pylori* positive control subjects and correlated to poor glycaemic control and the presence of DAN. Upregulation of eNOS expression could possibly represent a compensatory mechanism of gastric mucosa to induce rapid epithelial restitution in the presence of *H. pylori* infection; however, this mechanism seems to be impaired in gastric mucosa of diabetic patients. This observation indicates that NO plays a crucial role in impaired gastric mucosal defence of diabetic patients and diabetic gastropathy, partly due to attenuated expression of eNOS. Further studies however are needed to elucidate this field.

This work has been presented in an abstract form during the 15th UEGW, 27–31 October, Paris.

Practice points

- eNOS expression is attenuated in gastric corpus mucosa of *H. pylori* positive diabetic patients in comparison with *H. pylori* positive control subjects; this could possibly represent a compensatory mechanism of gastric mucosa to induce rapid epithelial restitution in the presence of *H. pylori* infection.
- eNOS attenuated expression is correlated to poor glycaemic control and the presence of Diabetic Autonomic Neuropathy. Protective eNOS action seems to be impaired in gastric mucosa of diabetic patients.

Research agenda

- To prospectively investigate whether poor glycaemic control results in gastric mucosal susceptibly and gastric inflammation.
- To elucidate the implication of eNOS in human gastric mucosal blood flow.
- To investigate whether gastric ulcer healing rate is impaired in diabetic patients with long-standing poor glycaemic control.

Conflicts of interest

None.

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REVIEW

Helicobacter pylori infection and endocrine disorders: Is there a link?

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Abstract

Helicobacter pylori (*H pylori*) infection is a leading world-wide infectious disease as it affects more than half of the world population and causes chronic gastritis, peptic ulcer disease and gastric malignancies. The infection elicits a chronic cellular inflammatory response in the gastric mucosa. However, the effects of this local inflammation may not be confined solely to the digestive tract but may spread to involve extra-intestinal tissues and/or organs. Indeed, *H pylori* infection has been epidemiologically linked to extra-digestive conditions and diseases. In this context, it has been speculated that *H pylori* infection may be responsible for various endocrine disorders, such as autoimmune thyroid diseases, diabetes mellitus, dyslipidemia, obesity, osteoporosis and primary hyperparathyroidism. This is a review of the relationship between *H pylori* infection and these endocrine disorders.

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Key words: *Helicobacter pylori*; Hormones; Thyroid; Osteoporosis; Diabetes; Dyslipidemia

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a gram-negative, spiral-shaped pathogenic bacterium that specifically colonizes the gastric epithelium and causes chronic gastritis, peptic ulcer disease and/or gastric malignancies[1,2]. The infection induces an acute polymorphonuclear infiltration in the gastric mucosa. If the infection is not effectively cleared, this acute cellular infiltrate is gradually replaced by an immunologically-mediated, chronic, predominantly mononuclear cellular infiltrate[3]. The latter is characterized by the local production and systemic diffusion of pro-inflammatory cytokines[4], which may exert their effect in remote tissues and organic systems[5]. As a result, *H pylori* infection has been epidemiologically linked to some extra-digestive conditions, including endocrine disorders (Table 1), although there are contradictory data regarding the relationship between *H pylori* infection and these diseases.

H pylori AND DIABETES MELLITUS

The relationship between diabetes mellitus (DM) and *H pylori* infection is controversial. According to some studies there is a high prevalence of *H pylori* infection in patients with either type I [6-9] or type II DM[10-13] which is correlated with the duration of DM[7,9], the presence of dyspeptic symptoms[13,14] and cardiovascular autonomic neuropathy[13,15], age[6,8], gender[16], body mass index (BMI)[16], blood pressure[16], fasting glucose[16] and the HbA1c levels[16]. In particular, the prevalence of *H pylori* infection was found to be higher in obese, female, middle-aged patients with a long standing DM, dyspeptic symptoms, cardiovascular autonomic neuropathy and increased blood pressure, fasting glucose levels and HbA1c values[6-9,13-16]. This could be related to a reduced gastric motility and peristaltic activity[10], various chemical changes in gastric mucosa following non-enzymatic glycosylation processes[10] and an impaired non-specific immunity observed in diabetic patients[11].

In contrast, other studies showed that *H pylori* infection is not associated with DM, as there is no

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difference in the prevalence of *H pylori* infection between diabetics and non-diabetics[17], regardless of the type[8,17-22] and duration of DM[18,19,22] and/or severity of dyspeptic symptoms in patients with DM[22]. The presence of micro-angiopathy in patients with DM may be a negative factor for colonization by *H pylori*, because micro-vascular changes in the gastric mucosa may create an unfavourable environment for the establishment or survival of *H pylori*[16]. Interestingly, one study even showed a lower sero-prevalence of *H pylori* in patients with DM, in comparison with the healthy population[23], while another showed a significantly lower incidence of *H pylori* infection in diabetics with active duodenal ulceration, as compared with non-diabetics[24].

The relationship between gastrointestinal symptoms in DM and *H pylori* infection is also controversial. According to some studies, there is no difference between diabetics and non-diabetics concerning the prevalence of *H pylori*-related gastro-duodenal disorders[17]. Moreover, *H pylori* infection was not associated with either delayed gastric emptying[9,25] or upper gastrointestinal symptoms in DM[19,21,25]. On the other hand, a high prevalence of esophagitis and peptic ulcer was found in *H pylori*+ve patients with DM, with or without dyspepsia, especially those with cardiovascular autonomic neuropathy[13,15] suggesting that this population should be considered as "high risk" for *H pylori* infection and suitable candidates for treatment[12]. In addition, some data demonstrated a higher prevalence of *H pylori* infection in diabetic patients with dyspepsia[14,26], reactive gastritis[27] and chronic gastritis[26] compared to those with no signs or symptoms of gastrointestinal disease.

The relationship between DM complications and *H pylori* infection is another issue which is contentious and deserves further investigation, as only few data are available. According to some data there is no relationship between *H pylori* infection and diabetic complications, such as nephropathy[13], retinopathy[13], and/or micro-angiopathy[16] while other data shows that virulent strains of *H pylori*, such as cytotoxin-associated gene $CagA^+$, are associated with macro-angiopathy[16], neuropathy[16] and micro-albuminuria in type Ⅱ diabetic patients, maybe due to an immuno-mediated injury at the level of the endothelium, caused by a systemic immune response to the infection, leading to albumin leakage[28]. Additionally, some data indicate a possible association of *H pylori* infection and the development of coronary heart disease, thrombo-occlusive cerebral disease, or both, in diabetic patients[29].

One point on which all studies seem to converge is that the effectiveness of eradication regimens for *H pylori* infection is significantly lower in diabetics than in non-diabetics[20,30-32] whereas re-infection rates seem to be higher, especially in patients with type Ⅱ DM compared to the general population[20,31]. This may be due to changes in the gastric microvasculature leading to reduced absorption of antibiotics. Alternatively, frequent antibiotic use in diabetics may result in the development of resistant *H pylori* strains[30,32]. Moreover, type I diabetic patients achieve lower *H pylori* eradication rates on standard triple therapy than non-insulin-dependent diabetic subjects, regardless of the dosage and/or the duration of therapy[20,31,32], and higher re-infection rates one year after eradication of *H pylori* compared with control subjects[33]. Quadruple therapies seem to cure a large percentage of patients who fail first-line therapy, although this is accompanied by a greater incidence of minor side effects[20,31]. These data suggest that vaccine development seems to be the only effective long term treatment for patients with DM[20].

Noteworthy is the observation that children with type I DM and *H pylori* infection had an increased daily insulin requirement compared with their uninfected peers[34]. Finally, several issues, such as the role of *H pylori* in etiopathogenesis of DM and the influence of *H pylori* eradication on the control of DM, remain to be elucidated.

Table 1 Endocrine disorders in relationship with *H pylori* infection

Endocrine disorders
Autoimmune thyroid diseases
Autoimmune atrophic thyroiditis
Hashimoto's thyroiditis
Thyroid mucosal associated lymphocyte tissue (MALT) lymphoma
Diabetes mellitus
Dyslipidemia
Obesity
Osteoporosis
Primary hyperparathyroidism

H pylori AND OSTEOPOROSIS

There are limited data regarding the association between *H pylori* infection and osteoporosis. According to one study, *H pylori* infection was not accompanied by significant changes in levels of markers of bone metabolism in children, such as estradiol, parathyroid hormone (PTH), cross-linked collagen I carboxy terminal telopeptide, total alkaline phosphatase (ALP), bone-specific ALP, N-terminal cross-links of human pro-collagen type I , osteocalcin, calcium and phosphate[35]. In another study, infection by $CagA^+$ *H pylori* strains was more prevalent in men with osteoporosis compared to the general population, who showed reduced systemic levels of estrogens and increased bone turnover[36]. *H pylori* infection by $CagA^+$ strains may therefore be considered a risk factor for osteoporosis in men[36]. Further studies are required to clarify the relationship between *H pylori* infection and osteoporosis and whether *H pylori* infection causes time-dependent changes in bone turnover markers during the long course of this chronic inflammatory disease.

H pylori AND HYPERPARATHYROIDISM

There are only a few studies attempting to clarify the association between *H pylori* infection and hyperparathyroidism. In fact, only one study showed that *H pylori* infection was more prevalent amongst patients with primary hyperparathyroidism (PHPT) than in the

general population, suggesting that patients with PHPT, and especially those with dyspeptic symptoms, should be evaluated for *H pylori* infection and treated appropriately if positive[37]. Also, a case report described an association of PHPT with duodenal ulcer and *H pylori* infection[38]. On the other hand, another study claimed no significant relationship between parathyroid abnormalities and *H pylori* infection in haemodialysis patients and this study found that a longer period of dialysis therapy was related to a decreased ability of these patients to produce antibodies against *H pylori*[39].

H pylori AND OBESITY

The relationship between obesity and *H pylori* infection is controversial. According to some studies, the risk of *H pylori* infection does not increase in overweight young persons[40] and *H pylori* seropositivity or CagA antibody status are not associated with the BMI[41,42] or fasting serum leptin levels[41]. Furthermore, one study indicated an inverse relationship between morbid obesity and *H pylori* seropositivity, leading to the hypothesis that the absence of *H pylori* infection during childhood may enhance the risk of the development of morbid obesity[43]. In contrast, other studies showed that obesity[10] and/or an elevation of the BMI[44] may be associated with an increased incidence of *H pylori* colonization, probably as a result of reduced gastric motility[10]. In addition, the incidence of *H pylori* infection in patients undergoing Roux-en-Y gastric bypass surgery for morbid obesity was higher than that found in all patients undergoing endoscopies and biopsy, even though the incidence of infection was not higher in controls matched for age[45].

The relationship between obesity and *H pylori* eradication is also controversial. There are data which demonstrate that eradication of *H pylori* significantly increases the incidence of obesity in patients with peptic ulcer disease, since it increases the level of BMI[46,47], and/or enhances the appetite of asymptomatic patients, due to an elevation of plasma ghrelin[48] and a reduction of leptin levels[49,50]. In fact, *H pylori* infection caused a marked reduction in plasma levels of ghrelin[44,49,51-53], as a result of a negative effect of this infection on the density of gastric ghrelin-positive cells[51,54] and an increase in plasma levels of leptin and gastrin[49,55,56]. Since ghrelin exerts orexigenic and adipogenic effects in contrast to leptin which exerts anorexigenic effects[52], alterations in plasma levels of gastric originated appetite-controlling hormones in children and adults infected by *H pylori* may contribute to chronic dyspepsia and loss of appetite[49]. Consequently, *H pylori* can be a "protective" factor against the development of becoming overweight[50]. In contrast, other studies showed that there are no differences in plasma ghrelin levels between *H pylori*+ve and *H pylori*-ve patients matched for age and BMI[57] and that successful eradication of *H pylori* had no effect on plasma ghrelin levels[44,57].

H pylori AND THYROID DISEASES

There have been controversial reports linking *H pylori* infection to thyroid disorders including autoimmune thyroid disorders (ATD) such as autoimmune atrophic thyroiditis[58] and Hashimoto's thyroiditis[59], or thyroid mucosal associated lymphocyte tissue (MALT) lymphoma[60].

Thus, some studies have reported an increased prevalence of *H pylori* infection in adults[58,61,62] and children[63] with ATD and a relationship between *H pylori* infection and the presence of high titers of thyroid auto-antibodies, such as anti-thyroglobulin (anti-Tg) and anti-thyroperoxidase (anti-TPO) antibodies[58,61,62] resulting in abnormalities of gastric secretory function[58]. It has also been suggested that $CagA^+$ *H pylori* strains increase the risk for ATD, especially in women, and that they are involved in the pathogenesis of Hashimoto's thyroiditis. This is based on the detection of monoclonal antibodies against Cag-A^+ *H pylori* strains which cross-react with follicular cells of the thyroid gland and also on the fact that *H pylori* strains possessing the Cag-A pathogenicity island carry a gene encoding for an endogenous peroxidase[61]. Moreover, the strong correlation between IgG anti-*H pylori* antibodies and thyroid auto-antibodies, as well as the observation that eradication of *H pylori* infection is followed by a gradual decrease in the levels of thyroid auto-antibodies[64], suggest that *H pylori* antigens might be involved in the development of autoimmune atrophic thyroiditis or that autoimmune function in this disease may increase the likelihood of *H pylori* infection[58]. One study showed a significant decrease of Free-T_3 and Free-T_4 in *H pylori*+ve subjects compared to *H pylori*-ve controls[62].

On the contrary, other studies showed no differences in the serum levels of thyroid hormones or thyroid auto-antibodies in patients with and without *H pylori* infection[59,65] whereas *H pylori* infection seemed not to increase the risk of ATD in individuals with dyspeptic symptoms[65]. Taking these results into account, it was proposed that screening for ATD in patients with a positive urea breath test is not indicated[65]. Other studies have failed to show any correlation between *H pylori* infection and ATD in children[66]. Moreover, the similar prevalence of *H pylori* infection, with or without $CagA^+$ strains, in patients with Hashimoto's thyroiditis and controls argues against a true association between *H pylori* infection and Hashimoto's thyroiditis[59]. To further explore the relationship between ATD and *H pylori* infection more clinical trials are required.

Lymphoid follicles in the gastric mucosa are common in ATD, and *H pylori* infection plays a causative role[67]. When an autoimmune disease such as ATD coexists with *H pylori* infection[68], *H pylori* may be involved in the pathogenesis of extra-gastric MALT lymphomas, such as thyroid MALT lymphoma, as shown by a case report describing a primary thyroid MALT lymphoma which occurred in an *H pylori*+ve patient with gastric cancer and Hashimoto's thyroiditis[60]. In this case, after subtotal gastrectomy, the thyroid lymphoma became smaller transiently and when the patient was treated with *H pylori* eradication therapy, the lymphoma completely disappeared. Nevertheless, *H pylori* organisms were not detected in the thyroid lymphoma tissue by polymerase

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chain reaction (PCR), questioning the role of *H pylori* in the development of extra-gastric MALT lymphoma in patients with an autoimmune disease[60]. In addition, one study suggested that patients with an autoimmune disease might not be optimal candidates for *H pylori* eradication, even in the case of an early stage gastric MALT lymphoma, since very few of these patients responded to an *H pylori* eradication therapy[68].

On the other hand, it is important to realize that patients with *H pylori*-related gastritis, atrophic gastritis, or both conditions required increased daily doses of T_4 than controls, suggesting that normal gastric acid secretion is necessary for effective absorption of oral T_4[69]. In addition, development of *H pylori* infection in patients treated with T_4 led to an increased serum level of thyrotropin (TSH), an effect that was nearly reversed after eradication of *H pylori* infection[69].

H pylori AND DYSLIPIDEMIA

H pylori infection may cause dyslipidemia, as it leads to elevated levels of total cholesterol[70,71], low-density lipoprotein cholesterol (LDL-c)[71,72], lipoprotein Lp(a)[71], apolipoprotein apo-B[73], triglyceride concentrations[72,74,75] and decreased levels of high-density lipoprotein cholesterol (HDL-c)[73-78] and apolipoprotein apoA-1 concentration in the blood[73,75]. In addition, plasma levels of cholesterol and LDL-c were significantly higher in *H pylori*+ve patients with ischemic stroke compared to *H pylori*-ve patients[70]. It was postulated that chronic *H pylori* infection may shift lipid profiles towards an atherogenic direction *via* the action of pro-inflammatory cytokines, such as interleukins 1 and 6 (IL-1 and IL-6), interferon-α (INF-α) and tumour necrosis factor-α (TNF-α). These cytokines are capable of affecting lipid metabolism in different ways, including activation of adipose tissue lipoprotein lipase, stimulation of hepatic fatty acid synthesis and influencing lipolysis[71,79]. This atherogenic modified lipid profile created by *H pylori* infection may increase the risk for cardiovascular and cerebrovascular diseases, by participating in the process of atherogenesis, especially when Cag-A^+ cytotoxic strains of *H pylori* are present[80,81], although other studies do not support this hypothesis[71,82,83].

According to other studies, *H pylori* infection did not cause any significant changes in plasma levels of total cholesterol[78,84], triglycerides[78,84], LDL-c[78,84] and Apo-B[78,85].

The relationship between dyslipidemia and *H pylori* eradication is also controversial. After one year of eradication of *H pylori* in patients with duodenal ulcers, a significant increase of HDL-c, apo-AI and apo-AII levels was observed in the study by Scharnagl *et al*[86]. Moreover, eradication of *H pylori* in healthy subjects seems to increase HDL-c and decrease LDL-c levels[78]. Also, 6 mo following successful eradication of *H pylori* infection the plasma levels of total cholesterol and LDL-c were found to be significantly lower than those in *H pylori*+ve controls and *H pylori*+ve patients with stroke[70].

In contrast, one study showed that eradication of *H pylori* is associated with minor lipid changes[84], while others showed a significant increase in the incidence of hyperlipidemia in patients with peptic ulcer disease, as serum total cholesterol and triglycerides were elevated in these patients after eradication of *H pylori*[46,47,87].

Table 2 Endocrine disorders and eradication of *H pylori*

Endocrine disorders	*H pylori* eradication
Autoimmune thyroid diseases	↓ of thyroid auto-antibodies[64] ↓ of thyrotropin in *H pylori*+ve patients treated with T4[69]
Diabetes mellitus	↓ in diabetics more than in non-diabetics[20,30-32] ↓ in type I diabetic patients on standard triple therapy more than non-insulin dependent diabetic subjects, regardless of the dosage and/or the duration of therapy[20,31,32]
Dyslipidemia	↑ of HDL-c, apo-AI and apo-AII levels in patients with duodenal ulcers, after 1 year[86] ↑ of HDL-c and ↓ LDL-c levels in healthy subjects[78] ↓ of total cholesterol and LDL-c after 6 mo in *H pylori*+ve controls and *H pylori*+ve patients with stroke[70] ↔ of lipids in patients submitted for endoscopy[84] ↑ of total cholesterol and triglycerides in patients with peptic ulcer disease[46,47] or without[87]
Obesity	↑ of BMI in patients with peptic ulcer disease[46,47] ↑ of the appetite of asymptomatic patients, due to ↑ of plasma ghrelin[48] and ↓ of leptin levels[49,50] ↔ of plasma ghrelin levels in subjects referred for upper gastrointestinal endoscopy[44,57]

BMI: Body mass index; HDL-c: High-density lipoprotein cholesterol; apo-AI: Apolipoprotein AI; apo-AII: Apolipoprotein AII; LDL-c: Low-density lipoprotein cholesterol; +ve: Positive.

CONCLUSION

Since the discovery of *H pylori*, a variety of studies, essentially epidemiological or therapeutic trials, case reports and others, have evaluated the potential direct or indirect involvement of this bacterium in the pathogenesis of various extra-gastric diseases or disorders, amongst them disorders of the endocrine system. A critical review of data published on these proposed associations suggests a strong link between dyslipidemia and *H pylori* infection, whereas increasing evidence emerges on the role of *H pylori* infection in thyroid autoimmune diseases. On the contrary, the association between *H pylori* infection and obesity, PHPT, DM and osteoporosis remains controversial, as evidence is hindered by the small numbers and methodological problems. Therefore, these associations should be interpreted cautiously. Although some evidence suggests that eradication of *H pylori* may lead to an improvement of many endocrine disorders, such as DM, dyslipidemia and autoimmune thyroid disease, excluding obesity (Table 2), more clinical trials are needed in order to confirm this beneficial effect. In conclusion, the causal association between *H pylori* infection and endocrine disorders is still controversial but worthy of further investigation since these diseases affect many people and have a great impact on human health and health economics[88].

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Cumulative *H. pylori* Eradication Rates in Clinical Practice by Adopting First and Second-Line Regimens Proposed by the Maastricht III Consensus and a Third-Line Empirical Regimen

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OBJECTIVES: **The European Helicobacter Study Group has recently issued the current concepts in the management of *Helicobacter pylori* infection (Maastricht III Consensus Report, 2005). The aim of the study was to examine the cumulative *H. pylori* eradication rates that can be achieved in clinical practice by adopting first and second regimens as proposed by the Maastricht III consensus and a third-line empirical levofloxacin-based regimen.**

METHODS: **H. pylori-positive patients were treated initially with a first-line eradication triple regimen consisting of omeprazole, amoxicillin, and clarithromycin and subsequently with a second-line quadruple regimen consisting of omeprazole, bismuth, metronidazole, and tetracycline. Finally, after two previous *H. pylori* eradication failures, patients received omeprazole, amoxicillin, and levofloxacin, as a third-line empirical strategy. The success rate was calculated by both intention-to-treat (ITT) and per protocol (PP) analyses.**

RESULTS: **In total, 540 consecutive *H. pylori*-positive patients received first-line treatment (omeprazole, amoxicillin, and clarithromycin). *H. pylori* were eradicated in 380 patients and 40 patients were withdrawn (ITT, 70.3%; PP, 76%). The remaining 120 *H. pylori*-positive patients received second-line treatment (omeprazole, bismuth, metronidazole, and tetracycline). *H. pylori* were eradicated in 83 patients and 7 patients were withdrawn (ITT, 69.1%; PP, 73.45%). Finally, the remaining 30 *H. pylori*-positive patients received third-line treatment (omeprazole, amoxicillin, and levofloxacin). *H. pylori* were eradicated in 21 patients and 0 patients were withdrawn (ITT, 70%; PP, 70%). Thus, out of 540 patients initially included in the study, *H. pylori* were eradicated in 484 patients, 47 were withdrawn, and only 9 remained positive. These results give 89.6% ITT and 98.1% PP cumulative *H. pylori* eradication rates.**

CONCLUSIONS: **By adopting first- and second-line regimens, as proposed by the Maastricht III consensus and a third-line levofloxacin-based empirical regimen, high cumulative *H. pylori* eradication rates can be achieved. Thus, a substantial number of cultures to determine sensitivity to antibiotics can be avoided with beneficial consequences concerning cost.**

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INTRODUCTION

Helicobacter pylori infection is the main cause of gastritis, gastroduodenal ulcer disease, and gastric cancer (1–3). Since its discovery, the European Helicobacter Study Group has convened the Maastricht Consensus conferences (4,5) to issue and update guidelines on *H. pylori* infection. The guidelines

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cover indications for management and treatment strategies. The most recent Maastricht Consensus conference (6) was held in Florence, Italy. According to these guidelines, in countries with clarithromycin resistance of less than 20%, *H. pylori*-positive patients should be treated initially with a first-line eradication triple regimen consisting of a proton pump inhibitor (PPI), amoxicillin, and clarithromycin and subsequently with a second-line quadruple regimen consisting of PPI, bismuth, metronidazole, and tetracycline. In Greece, the clarithromycin resistance rate is approximately 13% (7,8) and therefore in our study we were justified use of the above-mentioned first- and second-line therapeutic regimens. Furthermore, according to the Maastricht Consensus conference (6), after two previous *H. pylori* eradication failures, the therapeutic approach includes gastric biopsy culture for determination of sensitivity and resistance to antibiotics. However, in clinical practice, an approach such as this is impractical and costly. Therefore, newer triple treatments, with other antibiotics than those used in first- and second-line treatments, could be considered as strong candidates for empirical third-line treatments. Along these lines, mainly two other antibiotics, i.e., levofloxacin and rifabutin, have emerged in the treatment of *H. pylori* infection (6).

The aim of this study therefore was to examine the cumulative *H. pylori* eradication rates that can be achieved in clinical practice by adopting the first- and second-line therapies as proposed by the Maastricht Consensus conference (6) and a third-line empirical levofloxacin-based regimen.

METHODS

Patients

In this single center prospective study, over a 4-year period (2003–2006), 540 consecutive *H. pylori* infected patients (324 men, 216 women, age range: 18–75 years), eligible for *H. pylori* treatment, were enrolled. All enrolled patients were endoscoped initially and at that time *H. pylori* status was assessed by the rapid urease test and histology. Patients under the age of 18, with clinically significant associated conditions (hepatic, cardiorespiratory, or renal diseases, insulin-dependent diabetes mellitus, neoplastic diseases, or coagulopathy), previous gastric surgery and allergy to any of the drugs used were excluded from this study. All enrolled patients were given written instructions concerning therapy to achieve good compliance. Compliance with therapy was determined from the interview and the recovery of empty envelopes of medication. Compliance with therapy was defined as the intake of 100% of the medication prescribed. Incidence of adverse effects was evaluated by means of a specific questionnaire, at the time *H. pylori* eradication success or failure was confirmed.

The indications for *H. pylori* treatment are shown in **Table 1**. There were patients with various indications for *H. pylori* eradication such as peptic ulcer disease, non-ulcer dyspepsia, nonsteroidal anti-inflammatory drugs consumption, first-degree relatives of patients with gastric cancer, low-grade mucosa-associated lymphoid tissue lymphoma, idiopathic thrombopenic purpura, and a group of patients who were endoscoped for various reasons, were found positive for *H. pylori* infection and despite the fact that there was no strong indication for *H. pylori* eradication, expressed the wish to be eradicated after full explanation by the doctor. The study design flow chart is shown in **Figure 1**. Briefly patients were treated initially with a first-line eradication triple regimen consisting of omeprazole, 20 mg b.i.d.; amoxicillin, 1 g b.i.d.; and clarithromycin, 500 mg b.i.d. for 10 days. Subsequently, in case of treatment failure, a second-line quadruple regimen consisting of omeprazole 20 mg b.i.d.; bismuth subcitrate 300 mg q.d.s., metronidazole 500 mg t.i.d.,

Table 1. Groups of *H. pylori*-positive patients treated

Indication for *H. pylori* treatment	No. of patients (%)
Peptic ulcer disease	65 (12)
Non-ulcer dyspepsia	280 (51.8)
NSAIDs consumption	94 (17.4)
First-degree relatives of patient with gastric cancer	55 (10.2)
Low-grade MALT lymphoma	5 (0.9)
Idiopathic thrombopenic purpura	6 (1.1)
Patients wish	35 (6.5)
Total	540

MALT, mucosa-associated lymphoid tissue; NSAIDs, nonsteroidal anti-inflammatory drugs.

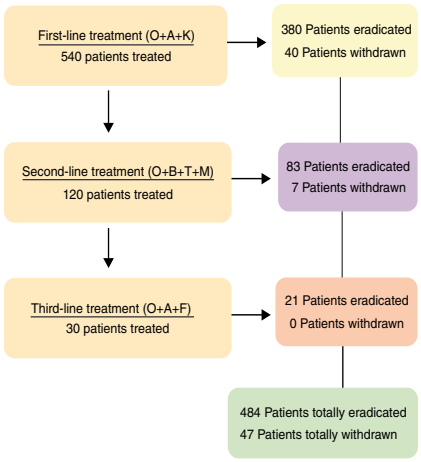
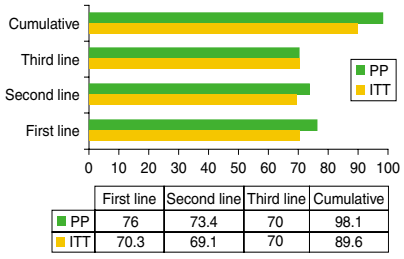


Figure 1. Flow chart summarizing results.

and tetracycline 500 mg b.i.d. (omeprazole, bismuth, metronidazole, and tetracycline) was prescribed for 10 days. Finally, after two previous *H. pylori* eradication failures, patients received omeprazole, 20 mg b.i.d.; amoxicillin 1 g b.i.d.; and levofloxacin 500 mg b.i.d. for 10 days, as a third-line empirical strategy. None of the medications were prescribed as part of a clinical trial and therefore there was no need for study approval by the ethical committee at our hospital. *H. pylori* eradication was checked by ^{13}C-urea breath test after stopping the prescribed regime for 4–8 weeks. To perform this test, 75 mg of ^{13}C-urea was used and citric acid was used as a test meal (9,10). Success rates, i.e., calculation of *H. pylori* eradication efficacy, were assessed by "intention-to-treat" (ITT) and "per protocol" (PP) analyses. Finally, 95% confidence intervals (CIs) were calculated for percentages.



	First line	Second line	Third line	Cumulative
PP	76	73.4	70	98.1
ITT	70.3	69.1	70	89.6

Figure 2. ITT and PP results (%) by first-, second-, and third-line therapies and also cumulative ITT and PP results.

RESULTS

The results are summarized in **Figure 1**. Out of 540 consecutive *H. pylori*-positive patients who received first-line treatment (omeprazole, amoxicillin, and clarithromycin), 500 completed the treatment successfully, taking all the medication prescribed. Of 540 patients, 40 were withdrawn due to either loss of follow-up ($n = 18$) or protocol violations ($n = 22$; compliance 95.8%, 95% CI: 93.7–97.3). *H. pylori* were eradicated in 380 patients giving 70.3% (95% CI: 66.5–74.2) ITT and 76% (95% CI: 72.2–79.4) PP results (**Figure 2**). The remaining 120 *H. pylori*-positive patients received second-line treatment (omeprazole, bismuth, metronidazole, and tetracycline) and 113 completed the treatment successfully, taking all the medication prescribed. Seven patients were withdrawn (five protocol violations and two losses of the follow-up; compliance 95.7%, 95% CI: 90.4–98.6). *H. pylori* were eradicated in 83 patients (ITT = 69.1%; 95% CI: 60.9–77.4; PP = 73.45%, 95% CI: 65.3–81.6; **Figure 2**). Finally, the remaining 30 *H. pylori*-positive patients received third-line treatment (omeprazole, amoxicillin, and levofloxacin). All patients completed the treatment successfully, taking all the medication prescribed (compliance 100%), and no patients were withdrawn. *H. pylori* were eradicated in 21 patients (ITT, 70%; PP, 70%, 95% CI: 53.6–86.4; **Figure 2**). Thus, out of 540 patients initially included in the study, *H. pylori* were eradicated in 484 patients, 47 were withdrawn, and only 9 remained positive. These results give 89.6% (95% CI: 87.6–92.2) ITT and 98.1% (95% CI: 96.9–99.3) PP cumulative *H. pylori* eradication rates (**Figure 2**). Stratification of results according to indication for treatment is shown in **Table 2**.

No severe side effects were reported. Mild adverse effects were reported by 18% (95% CI: 14.8–21.5) of patients receiving the first-line treatment, by 16.1% (95% CI: 10–24) of patients

Table 2. Stratification of results according to indication for treatment

Groups for *H. pylori* treatment	Patients eradicated (*n*)	Patients withdrawn (*n*)	Patients not eradicated (*n*)	ITT (%)	PP (%)
Peptic ulcer disease (*n*=65)	60	5	0	92.3	100
Non-ulcer dyspepsia (*n*=280)	261	15	4	93.2	98.4
NSAIDs consumption (*n*=94)	80	12	2	85.1	97.5
First-degree relatives of patient with gastric cancer (*n*=55)	45	8	2	81.8	95.7
Low-grade MALT lymphoma (*n*=5)	5	0	0	100	100
Idiopathic thrombopenic purpura (*n*=6)	6	0	0	100	100
Patient's wish (*n*=35)	27	7	1	77.1	96.4
Total (540)	484	47	9	89.6 (95% CI: 87.6–922)	98.1 (95% CI: 96.9–99.3)

ITT, intention to treat; MALT, mucosa-associated lymphoid tissue; NSAIDs, nonsteroidal anti-inflammatory drugs; PP, per protocol.

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receiving the second-line treatment, and by 20% (95% CI: 7.7–38.6) of patients receiving the third-line treatment.

DISCUSSION

The European Helicobacter Study Group founded in 1987 to promote multidisciplinary research into the pathogenesis of *H. pylori* has organized successful annual meetings and arranged task forces on paediatric issues and clinical trials. In addition, consensus meetings (4–6) have been convened on who, how, and when to treat patients with *H. pylori* infection. The matter of how to treat *H. pylori*-positive patients remains a challenge as, after more than 20 years of experience in *H. pylori* treatment, the ideal regimen to treat this infection has still not been found. Thus, even with the current most effective treatment regimens, including PPIs plus two antibiotics, usually clarithromycin and amoxicillin, in approximately 20% of patients the infection will not be eradicated and these patients will remain *H. pylori* positive (11). The quadruple combination of PPI, bismuth, tetracycline, and metronidazole has been recommended (6), even though bismuth is not available worldwide, and some National Guidelines have been accordingly changed (12). However, the quadruple combination still fails to eradicate *H. pylori* in approximately 20–30% of the cases. These cases constitute a therapeutic dilemma, as this means that patients who are not cured with the two above-mentioned treatments, which include clarithromycin and metronidazole, are resistant to either one or both of these drugs (11). Currently, a standard third-line therapy is lacking, and European guidelines (6) have recommended culture in these patients to select a third-line treatment according to microbial sensitivity to antibiotics. However, cultures are usually carried out only in research centres, and the use of this procedure as "routine practice" in patients who have failed several treatments is not feasible (13,14). Therefore, the evaluation of drugs, without resistance to nitroimidazole or macrolides, as components of retreatment combination therapies seems to be worthwhile. Levofloxacin, a fluoroquinolone antibacterial agent, with a broad spectrum of activity against Gram-positive and Gram-negative bacteria and proven efficacy in the treatment of infections of the respiratory tract, genitourinary tract, and skin (15), seems to be promising. Indeed, recent studies have shown good efficacy and tolerability of a levofloxacin-based regimen in patients with two consecutive *H. pylori* eradication failures (16–19).

In this study, we examined the *H. pylori* eradication rates that can be achieved in clinical practice by adopting the first- and second-line regimens as proposed by the Maastricht Consensus conference (6) and a third-line levofloxacin-based empirical regimen. The study was performed in Greece where clarithromycin resistance is approximately 13% (7,8). Therefore, we were entitled to start with the triple combination of PPI, clarithromycin, and amoxicillin, given that the threshold of clarithromycin resistance at which this antibiotic should not be used is 15–20% (6). The ITT and PP success rates of 70.3% and 76% for first-line, 69.1% and 73.45% for second–line, and 70% for third-line treatments were similar to success rates achieved in various other studies from countries with comparable antibiotic resistance(6,20–22). Alternatives to third-line rescue therapies other than levofloxacin-based regimens have been suggested. Thus, a rifabutin-based rescue therapy also constitutes a possible strategy after previous multiple eradication failures. However, rifabutin is expensive, it is associated with myelotoxicity (23), and, most importantly, it is considered to be a useful antimicrobial drug for patients with tuberculosis and its widespread use should be limited to prevent development of resistance. In contrast, levofloxacin is generally well tolerated, and most adverse events associated with its use are mild to moderate in severity and are transient (6).

By adopting first- and second-line regimens proposed by the Maastricht III consensus and an empirical levofloxacin-based third-line regimen, the cumulative *H. pylori* eradication rates reached the high levels of 89.6% (ITT) and 98.1% (PP). According to these results, a substantial number of cultures to determine sensitivity to antibiotics can be avoided with beneficial consequences concerning cost, as the cost of culture and *H. pylori* sensitivity test to antibiotics is cut out.

Some other studies have evaluated different regimens after failure of two or more eradication treatments and have also achieved a high overall eradication rate (24–29). In particular, a very recent study (30) evaluated the efficacy of different "rescue" therapies empirically prescribed over 10 years to 500 patients in whom at least one eradication regimen had failed to cure *H. pylori* infection, achieving similar results to ours. All the above emphasize the recommendation that in designing a treatment strategy we should use these regimens consecutively to achieve high success rates.

In summary, high cumulative *H. pylori* eradication rates can be achieved in clinical practice, by adopting first- and second-line regimens, as proposed by the Maastricht III consensus and a third-line levofloxacin-based empirical regimen. Thus, a substantial number of costly cultures to determine sensitivity to antibiotics can be avoided.

CONFLICT OF INTEREST

Guarantor of the article: Theodore Rokkas, MD, PhD, FACG, AGAF, FEBG.

Specific author contributions: Theodore Rokkas had the original idea for the study. He designed and organized the protocol, performed the statistical analysis of the data, and wrote the paper. All the authors treated and included patients in the study, had access to the data and the statistical analysis report, critically reviewed the paper, approved the final article, and attested to the validity of the results.

Financial support: This was an investigator-initiated unfunded study. All authors had access to the data and the statistical analysis report. Each author approved the final article and attested to the validity of the results.

Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

✓ In approximately 20% of patients, infection with *H. pylori* will not be eradicated and these patients will remain *H. pylori* positive.

✓ Currently, culture is recommended in these patients to select a suitable treatment according to microbial sensitivity to antibiotics. However, cultures are usually carried out only in research centers, and the use of this procedure as "routine practice" is not feasible.

WHAT IS NEW HERE

✓ By adopting first and second regimens, as proposed by the Maastricht III consensus, and a third-line levofloxacin-based empirical regimen, high cumulative *H. pylori* eradication rates can be achieved.

✓ Thus, a substantial number of cultures to determine sensitivity to antibiotics can be avoided with beneficial consequences concerning cost.

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ORIGINAL ARTICLE

Intrafamilial Spread of *Helicobacter pylori* Infection in Greece

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Goal: To find out the role of family members in the *Helicobacter pylori* infection in childhood by investigating the incidence of infection within families of *H. pylori*-infected children.

Background: *H. pylori* infection is usually acquired in early childhood and possibly family members are the main source of infection.

Study: One hundred consecutive children with upper gastrointestinal symptoms, without previous *H. pylori* eradication treatment were prospectively studied by gastroscopy and ^{13}C-urea breath test. Simultaneously, all family members were studied by ^{13}C-urea breath test regardless of earlier eradication treatment for *H. pylori* infection. The age of children and their parents, socioeconomic status, parents' education, and living conditions were recorded.

Results: Forty-four index symptomatic children were infected by *H. pylori*. No statistical difference was found concerning demographic factors, between *H. pylori*-positive and *H. pylori*-negative index children except age, which was higher in the *H. pylori*-infected children ($P = 0.009$). In all *H. pylori*-positive and in 71.4% of the negative index children, at least 1 more family member was infected ($P < 0.001$), always including a parent in the *H. pylori*-positive, compared with 69.6% in the *H. pylori*-negative group ($P < 0.001$). The percentage of infected siblings, mothers and fathers was higher in *H. pylori*-infected index children ($P < 0.001$, $P = 0.001$, and $P = 0.035$, respectively).

Conclusions: The prevalence of *H. pylori* infection is significantly higher among families of infected index children. The presence of at least 1 infected family member in all *H. pylori*-positive index children suggests that the family could be the main source of *H. pylori* infection in children.

Key Words: *H. pylori*, children, intrafamilial transmission

(*J Clin Gastroenterol* 2009;43:711–715)

About half of the population worldwide is estimated to be infected with *H. pylori*, which is more prevalent in low-income areas of the world. The exact mode of *H. pylori* transmission is yet to be established. The route of transmission is uncertain, but the oral-oral and fecal-oral routes are likely possibilities.[1] Close personal contact among family members appears to be a key factor[1,2]. Social and economic development decreases the prevalence, as reflected in comparisons both within and between countries. The infection is usually acquired in early childhood[3,4] and once established commonly persists throughout life, unless treated. Person-to-person transmission within family appears to be the predominant mode of contamination, particularly from mothers to children and among siblings, indicating that intimate contact is important.

To further elucidate the intrafamilial transmission of *H. pylori* infection in Greece, we prospectively investigated the occurrence of infection in all family members of each index symptomatic child.

MATERIALS AND METHODS

One hundred consecutive children, 94 of Greek and 6 of Albanian origin, with upper gastrointestinal symptoms, that is, epigastric or abdominal pain, vomiting and upper gastrointestinal bleeding were prospectively investigated by upper gastrointestinal endoscopy and carbon 13-urea breath test (^{13}C-UBT) of INFAI company (GmbH Bochum, Germany, *Helicobacter* INFAI test). None of them had received any earlier eradication therapy for *H. pylori* infection. Children who had received any antibiotic over the last 3 months were excluded from the study. The positivity of *H. pylori* infection of the index patients was confirmed by at least 2 of the following tests: culture, histology, rapid urease test, ^{13}C-UBT.

All family members of each index patient were simultaneously investigated by ^{13}C-UBT regardless of earlier eradication treatment for *H. pylori*. In 3 siblings who were less than 2 years old, a polyclonal stool test (Premier HpSA, Meridien Diagnostics) was performed instead of ^{13}C-UBT. All parents signed a written consent form before participating in the study. The cutoff value of ^{13}C-UBT was according to that recommended by INFAI Test (a change of $> 4\%$ in the value of ^{13}C-UBT over base line was considered positive).

The age of children and their parents, socioeconomic status, parents' education and living conditions were studied. Socioeconomic status was defined according to parental occupation as upper middle (eg, professionals with a college education as doctors, lawyers, university professors, businessmen), middle (eg, lower paid white collar workers but not manual laborers, primary and high school teachers, technicians, nurses, small business owners) and lower class (eg, manual laborers, peasants, unemployed). Parental education was expressed as the total years of studies (up to 6 y elementary, 12 y including high school, and above 12 y following attendance at college or university). Parents' and siblings' medical history concerning

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previous investigations and treatment for *H. pylori* infection was recorded. *H. pylori* status of each family member was estimated according to the ^{13}UBT values (current *H. pylori* status).

For those family members with a negative ^{13}C-UBT, who had received eradication therapy in the past (after index patient's birth) the positivity was assessed by histology, thus providing additional information concerning earlier *H. pylori* family status. We took into account eradication treatment in family members offered at any time after the index child's birth, but we did not examine the family status before or at birth.

Statistical Analysis

Comparisons of categorical variables were undertaken by the application of χ^2, Fisher exact or Mann-Whitney tests. Student *t* test for independent samples was used for comparisons of quantitative variables and *t* test for paired samples was used to compare the differences between parents. Pearson correlations and their significance were also calculated, odds ratios and 95% confidence intervals were given. All tests were 2-tailed at a significance level < 0.05.

RESULTS

H. pylori Status Within Family at the Time of the Study

Forty-four (index cases) out of 100 symptomatic index children were infected by *H. pylori* (Table 1). The mean age of infected index children was 11.02 ($\pm$ 3.24) years, compared with 9.39 ($\pm$ 2.90) years for the uninfected controls ($P < 0.009$). Notably, the infected and uninfected children had no difference in symptomatology. A total of 281 (197 parents, 84 siblings) family members were investigated. The mean age of siblings was 11 years (range: 1 to 21 y). In 3 parents, the ^{13}C-UBT was not performed (1 mother and 1 father had died and 1 father had abandoned the family before the index child's birth), whereas 3 additional siblings were not investigated by ^{13}C-UBT because of their young age (8, 14, and 23 mo, respectively). In those 3 siblings, the polyclonal stool test was negative. Among the relatives of the 100 index patients, 56 of 99 (56.6%) mothers, 50 of 98 (51.0%) fathers, and 20 of 84 (23.8%) siblings were infected. No significant difference in symptomatology between infected and uninfected parents was found.

Socioeconomic Status and Living Conditions

Differences between *H. pylori*-infected index symptomatic children and controls (*H. pylori*-negative symptomatic index children) with respect to sex, family socioeconomic status, number of children at home were all nonsignificant as shown in Table 1. Regarding nationality, the difference between infected and uninfected index children was significant ($P = 0.006$). All 6 non-Greek infected index children were Albanian immigrants, their parents were manual laborers and belonged to the *H. pylori*-positive group. No significant differences were noticed between cases and controls concerning parental age and education, age of first sibling as well as sharing room or bed with parents or siblings (data not included).

Table 1. Sociodemographic Characteristics

	Total n (%)	Cases n (%)	Controls n (%)	*P*
Sex				
Male	48 (48)	21 (47.7)	27 (48.2)	NS
Female	52 (52)	23 (52.3)	29 (51.8)	
Socioeconomic status				
Upper middle	39 (39.4)	18 (41.9)	21 (37.5)	
Middle	39 (39.4)	15 (34.9)	24 (42.9)	NS
Lower	21 (21.2)	10 (23.3)	11 (19.6)	
Nationality				
Greek	94 (94.0)	38 (86.4)	56 (100.0)	0.006
Albanian	6 (6.0)	6 (13.6)	0	
Children in home				
One	27 (27.0)	9 (20.5)	18 (32.1)	NS
Two	62 (62.0)	30 (68.2)	32 (57.1)	
Three	10 (10.0)	5 (11.4)	5 (8.9)	
Four	1 (1.0)	0 (.0.0)	1 (1.8)	

NS indicates not significant.

^{13}C-UBT δ-value

The mean total ^{13}C-UBT δ-value of mothers as well as of fathers did not differ significantly between the 2 groups, but ^{13}C-UBT δ-values of mothers were found to be higher compared with those of fathers in both infected [$t(40) = 3.372$, $P = 0.002$] and noninfected children [$t(54) = 3.005$, $P = 0.004$], following the application of the 2-tailed paired *t* test. ^{13}C-UBT δ-values of mothers were not found to correlate with those of fathers in either group (Pearson $r = 0.060$, $n = 41$, $P = 0.711$, for the group of infected children and $r = 0.177$, $n = 55$, $P = 0.196$ for the group of noninfected children). The percentage of mothers with positive ^{13}C-UBT was higher among the infected children (Table 2) compared with the noninfected (72.1% and 44.6%, respectively, $P < 0.006$) children. This difference was not found to be significant for fathers (58.1% vs. 45.5%, $P = 0.213$). At least 1 infected sibling was found in 51.45% of the *H. pylori*-positive index children compared with 5.3% of the *H. pylori*-negative ($P < 0.001$, odds ratio: 19.06). In the infected index children group, at least 1 more member was infected in 93.2% of the cases compared with 65% of the noninfected ($P < 0.001$) children. Two or more family members were found to be infected significantly more often among the infected index children group than among the noninfected group (60.5% vs. 28.6%, $P < 0.001$).

Correlations of infected children's bacterial load (expressed by ^{13}C-UBT δ-value) with various indices of family bacterial load such as that of mother, father, maximum or total load of siblings, maximum family members' or total family members' bacterial load were all nonsignificant.

H. pylori Status of the Family Before Eradication

In 23 family members (14 of infected and 9 of the control index children) with negative ^{13}C-UBT at the time of the study, eradication therapy had been applied 2 to 29 months before the study (in all cases after index patient's birth). Taking into account the *H. pylori* infection status before eradication, all indices of family infection were

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Table 2. Helicobacter pylori-infected Family Members According to ^{13}C-UBT at the Time of the Study

Member	Cases (%)	Controls (%)	*P**	OR	95% CI for OR
Mother	31/43 (72.1)	25/56 (44.6)	0.006**	3.20	1.37–7.49
Father	25/43 (58.1)	25/55 (45.5)	0.213	1.67	0.74–3.73
At least 1 parent	39/44 (88.6)	36/55 (65.5)	0.007**	4.12	1.39–12.17
Both parents infected	17/42 (40.5)	14/55 (25.5)	0.116	1.99	0.84–4.73
At least 1 sibling	18/35 (51.4)	2/36 (5.3)	< 0.001**	19.06	3.96–91.66
At least 1 other family member	41/44 (93.2)	36/55 (65.5)	0.001**	7.21	1.97–26.40
Two or more other family members	26/43 (60.5)	16/56 (28.6)	0.001**	3.82	1.65–8.88

*Pearson χ^2.
**Two-tailed significance at level 0.01.
CI indicates confidence interval; ^{13}C-UBT carbon 13-urea breath test; OR, odds ratio.

found significantly more often among *H. pylori*-positive index children compared with controls (Table 3).

DISCUSSION

This prospective study provides evidence that family could be the main source of *H. pylori* infection in children of the population studied, as in all families of infected index children at least 1 more member was infected. The advantage of this study is that all family members were included as a study sample and for the detection of infection the most sensitive, a noninvasive test (^{13}C-UBT) was used. The test enabled a detailed examination of the relationship between *H. pylori* infection and intrafamilial dynamics, allowing accurate conclusions to be made.

The fact that transmission of *H. pylori* occurs mainly from parent to child, and not vice versa, is supported by the observation that *H. pylori* infection predominantly occurs in childhood.[2–7] The major route of transmission remains poorly understood. Person-to-person transmission within family seems to be the predominant mode, particularly from mothers to children and among siblings[8]. This is indicative that intimate contact is important.

There is increasing evidence supporting the intrafamilial transmission of *H. pylori* infection. Rothenbacher et al[5] in a population-based study aimed to determine the role of parental infection status in the transmission of *H. pylori*; parental infection status was determined by the measurement of *H. pylori* immunoglobulin G antibodies in the saliva, which has lower sensitivity and specificity compared with the ^{13}C-UBT used in this study. Even in that case, a higher prevalence of infection was also found when parents (especially mothers) had positive saliva antibodies. Sex, number of siblings, and parental education did not demonstrate any significant association with *H. pylori* status of the child, as was also found in our study.

Children living in developing countries present several risk factors for acquisition, including young age, poor sanitation, overcrowding, low maternal education, low socioeconomic status, and recurrent gastroenteritis[7,9,10]. In this study these risk factors were not identified. Several independent population-based cross-sectional studies in South Germany[2], Turkey,[11] and Vietnam[12] have consistently shown that parents may play a key role in transmitting *H. pylori* to the child, and these findings are confirmed by this study. Ito et al[13] attempted to determine the seroprevalence of *H. pylori* infection within family units of Japanese Brazilians and to identify the risk factors associated with intrafamilial transmission. The prevalence of infection was 16% for those with seropositive parents compared with 3.5% with uninfected parents. The role of the family in *H. pylori* transmission has also been shown by molecular techniques based on gene sequencing, where identical strains among parents and siblings were found.[4,9,14,15]

The main role of the mother in *H. pylori* transmission has been confirmed by several investigators. Two seroepidemiologic studies, 1 from a developed country (Sweden)[6] and 1 from an underdeveloped region (Benin/Africa)[16] demonstrated a strong familial clustering of *H. pylori* infection. In both studies, an infected mother was a strong determinant for child infection. In a large community-based birth cohort longitudinal study it was suggested that infected mothers were the main source of infection for

Table 3. *Helicobacter pylori* Status of the Family Before Eradication

Member	Cases (%)	Controls (%)	*P**	OR	95% CI for OR
Mother infected	36/43 (83.7)	28/56 (50.0)	0.001***	5.14	1.96–13.49
Father infected	33/43 (76.7)	31/55 (56.4)	0.035**	2.55	1.05–6.19
At least 1 parent	44/44 (100)	39/56 (69.6)	< 0.001***	—	—
Both parents infected	25/42 (59.5)	19/55 (34.5)	0.014**	2.79	1.21–6.39
At least 1 sibling	19/35 (54.3)	2/36 (5.3)	< 0.001***	21.37	4.44–102.90
At least 1 other family member	44/44 (100)	40/56 (71.4)	< 0.001***	—	—
Two or more other family members	32/43 (74.4)	20/55 (36.4)	< 0.001***	5.09	2.12–12.25

*Pearson χ^2.
**Two-tailed significance at level 0.05.
***Two-tailed significance at level 0.01.
CI indicates confidence interval; OR, odds ratio.

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their children.[14] Fujimoto et al[17] in a recent study in Japan found that although *H. pylori* infection had decreased in the general population over the last decade, it did not differ in young children, most likely because of mother-to-child transmission. In a prospective study Rowland et al[3] found that an infected mother, an infected older sibling and delayed weaning, were all risk factors for infection. Transmission may occur by the common use of spoons, the licking of pacifiers or the teats of feeding bottles or even by premastication of food when mothers feed their children.[18,19] In another study when ^{13}C-UBT was used for *H. pylori* identification, the mother's role was identified as more significant although the study sample did not include all fathers but only those who accompanied the child at endoscopy.[2] The mother's symptoms of nausea and vomiting were significantly associated with the risk of *H. pylori* infection for children[13] mainly through contact with regurgitated gastric juice in the mother's mouth. In this study no correlation with the mother's symptoms was revealed.

The much stronger impact of maternal *H. pylori* status on the child's infection was also confirmed in this study by assessing the bacterial load. The maternal bacterial load was higher compared with that of the fathers in both the groups, suggesting an additional reason for the high risk of the infected mother—compared with the infected father—for the transmission of the infection to the child. However, no significant correlation of the bacterial load of index child with the bacterial load of mother, father, maximum load of siblings, or total bacterial load of the family (expressed indirectly by ^{13}C-UBT δ-value) was found. In this study the risk of infection increased significantly in accordance with the number of infected persons in a child's family, as was also found in an earlier study.[9]

The presence of infected siblings was also an independent risk factor for the infection in children from studies conducted in high-prevalence countries.[20] Sharing a bed or bedroom with an infected sibling in early childhood significantly increases the risk of childhood *H. pylori* infection.[21] The high rate of reinfection of adolescents, especially having siblings younger than 5 years strengthens the theory of transmission of the infection among siblings.[22] Indeed, in this study infected siblings seemed to play an important role—having an odds ratio of 21.7—for *H. pylori* infection. The polyclonal stool antigen test performed in the 3 young siblings has, just like the UBT, a low sensitivity and specificity in this age group.[23] However, in this age group the prevalence of infection is low and the number of siblings is perhaps too small to have a significant effect on the results. Although no correlation with sharing the same room or bed with infected siblings was found, the transmission could be explained by the common use of spoons or cups or from another common source of infection. On the contrary, Weyermann et al[24] in a prospective birth cohort study found no evidence that transmission among siblings was relevant.

We did not have information relating to parental status at birth, although it is assumed that the positive members expressed by the current status were also positive at the index child's birth. In contrast, parents with a negative status at the time of the study could have been eradicated many years before the child's birth. For this reason, we included only the family members identified as positive and treated after the index child's birth.

Studies concerning the prevalence of *H. pylori* infection in asymptomatic adults and children in Greece are limited. Serologic studies showed a range from 15.5% to 39.4% in children,[25,26] whereas in asymptomatic adults aged above 20 years the prevalence was 59%[25] and 70% in blood donors aged 20 to 50 years.[26] More recent data indicate a decline in prevalence.[27,28] Among 259 asymptomatic children aged 1 to 15 years the seroprevalence was 14.7%, with an increase according to age from 9.7% in those below the age of 5 years, 15.3% from 6 to 10 years, and up to 21.1% in children aged 11 to 15 years (unpublished data).

According to our findings, household clustering of *H. pylori* infection is clearly demonstrated. Not only infected mother or siblings but also infected father are a risk factor for infection of the child within the family. However, the odds ratio is higher for infected siblings and mother compared with that of the father. The identification in every family of at least 1 infected parent enhances the central role of parents for the *H. pylori* infection in childhood. Finally, the fact that in every infected index child at least 1 other person within the family was also infected, may lead to the conclusion that the family possibly plays the main role in the transmission of *H. pylori* infection to the child.

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CagA and VacA Polymorphisms Do Not Correlate with Severity of Histopathological Lesions in *Helicobacter pylori*-Infected Greek Children▽†

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The presence of various numbers of EPIYA tyrosine phosphorylation motifs in the CagA protein of *Helicobacter pylori* has been suggested to contribute to pathogenesis in adults. In this prospective study, we characterized *H. pylori* isolates from symptomatic children, with reference to the diversity of functional EPIYA motifs in the CagA protein and *vacA* isotypes, and assessed the potential correlation with the histopathological manifestations of the infection. We analyzed 105 *H. pylori* isolates from 98 children and determined the diversity of EPIYA motifs in CagA by amplification and sequencing of the 3′ variable region of the *cagA* gene as well as *vacA* isotypes for the signal, middle, and intermediate regions. CagA phosphorylation and levels of secreted IL-8 were determined following in vitro infection of AGS gastric epithelial cells. Histopathological evaluation of *H. pylori* colonization, activity, and severity of the associated gastritis was performed according to the updated Sydney criteria. EPIYA A (GLKN[ST]EPIYAKVNKKK), EPIYA B (Q[V/A]ASPEPIY[A/T]QV AKKVNAKI), and EPIYA C (RS[V/A]SPEPIYATIDDLG) motifs were detected in the ABC (46.6%) and ABCC (17.1%) combinations. No isolates harboring more than two EPIYA C motifs in CagA were found. The presence of isogenic strains with variable numbers of CagA EPIYA C motifs within the same patient was detected in seven cases. Occurrence of increasing numbers of EPIYA C motifs correlated strongly with presence of a high-vacuolation (s1 or s2/i1/m1) phenotype and age. A weak positive correlation was observed between vacuolating *vacA* genotypes and presence of nodular gastritis. However, CagA- and VacA-dependent pathogenicities were not found to contribute to severity of histopathology manifestations in *H. pylori*-infected children.

Helicobacter pylori infects 50% of the world's population, and wide differences in prevalence of infection appear to exist between countries with different levels of socioeconomic development. Infection usually occurs in childhood and in the majority of cases remains asymptomatic, although major reasons for endoscopy referral can include recurrent epigastric or abdominal pain, with or without vomiting, neither of which correlates with *H. pylori* infection (17). Antral nodularity is a well-described endoscopic feature of *H. pylori*-infected children, and histological observations usually include superficial chronic active gastritis with occasional infiltration of eosinophils; in far fewer cases, they include peptic ulcers; and very rarely, they include gastric atrophy and intestinal metaplasia (13, 27). If the infection is left untreated, it persists through adulthood, and although it can still remain asymptomatic in the vast majority of infected hosts, *H. pylori* infection is now regarded as the most important etiological risk factor for development of gastric cancer in developed countries (28). *H. pylori* pathogenesis is manifested through a combined effect of bacterial virulence factors, host genetics, and environmental factors, which orchestrate toward the development of distinct phenotypes in adults, namely, superficial asymptomatic gastritis, duodenal ulcer, and gastric cancer (3). The expression and translocation of cytotoxin-associated gene antigen (CagA), a putative *H. pylori* virulence factor, inside gastric epithelial cells by *cagA*-positive *H. pylori* strains harboring a functional type IV secretion system has been suggested to play an important role in *H. pylori* pathogenesis (22). Early epidemiological studies of adults associated the presence of the *cagA* gene with development of peptic ulcer disease (31); gastric cancer (14); and increased inflammation (35), cellular proliferation (36), and intestinal metaplasia (20) of the gastric mucosa. However, in infected children, neither *cagA* status nor any other putative *H. pylori* virulence factor has been found to correlate with clinical outcome or severity of histological manifestations. However, recent advances into the fascinating cellular biology of CagA inside the gastric epithelial cell have enhanced its reputation as a potential bacterial oncoprotein (22). Following its translocation inside the gastric epithelial cell via the type IV secretion system (32), CagA has been shown to become at least partly tyrosine phosphorylated (5, 11, 41) by Src family kinases (42, 44) on repeating 5-amino-acid glutamic-proline-isoleucine-tyrosine-alanine (EPIYA) motifs present at the C terminus of the protein. Analysis of EPIYA motifs in CagA has

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revealed considerable type variation, depending on the peptide sequence surrounding it, namely, EPIYA A (EPIYAKVNK KK), EPIYA B (EPIYAQVAKKV), or EPIYA C (EPIYATI DDLG) in isolates from Western populations or EPIYA D (EPIYATIDFD) in isolates of Asian origin. In addition, considerable variation in number of repeating EPIYA C or D motifs at the carboxyl terminus of the protein (10, 44) has been observed, and biological activity of CagA was suggested to be determined by variation in these motifs (25) in phosphorylation-dependent as well as -independent ways (23). Hence, the number and type of EPIYA phosphorylation motifs may be viewed as putative virulence determinants of CagA activity and therefore become useful clinical markers that may predict the degree of individual *H. pylori* strain virulence potential. In this context, we proposed a PCR amplification and sequencing-based strategy for accurate characterization of the number and type of EPIYA motifs of CagA in *H. pylori* clinical isolates (34).

A multifactorial role has also been attributed to the secreted VacA virulence factor (16), a protein with multiple cellular activities, as it can disrupt endocytic trafficking of host cells, promote cell death through apoptosis, suppress the local immune system, and possibly potentiate the development of ulcers (6). Although the *vacA* gene is present in all *H. pylori* strains, it contains at least three variable parts, the s region, the i region, and the m region, which encode the signal, intermediate, and middle peptides, respectively, which have all been classified as allelic types 1 and 2. The s1-or-s2/i1/m1-or-m2 and s1-or-s2/i1-i2/m1-or-m2 VacA isotypes induce, in general, high and moderate levels of vacuolation, respectively, whereas the s1-or-s2/i2/m1-or-m2 strains induce very little or no vacuolation (39). Consequently, the *vacA* s/m genotype can also be regarded as a marker of pathogenicity of individual strains (8). Moreover, phylogenetic linkage analysis studies have indicated that there may be a functional basis for the selection of *vacA* and *cagA* isotypes (50), although there is substantial distance between *vacA* loci and *cag* genes on the bacterial genome. Furthermore, the intermediate region has been associated with development of gastric cancer (39).

In the present study, we investigated the potential association of the CagA and VacA virulence factor polymorphisms with clinicopathological manifestations of the disease in symptomatic Greek children. More specifically, *H. pylori* clinical strains isolated from symptomatic children were characterized with regard to the number and type of repeating EPIYA phosphorylation motifs in CagA protein and the *vacA* signal, intermediate, and middle region genotypes. Furthermore, these clinical isolates were carefully assessed for their ability to express phosphorylated CagA as well as induce interleukin-8 (IL-8) secretion following infection of gastric epithelial cells. Finally, the potential association of such functional bacterial determinants with *H. pylori*-associated histopathology in these patients was assessed.

MATERIALS AND METHODS

Patients. The study included 98 symptomatic *H. pylori*-infected children, 2 to 16 years old, who underwent upper endoscopy at the Gastroenterology Clinic of the First Department of Pediatrics of Athens University, Aghia Sophia Children's Hospital. All children were Greek in origin, and their parents had given their consent for participation in the study. The study (protocol number 19321/13.09.2007) was approved by the Hospital Scientific Committee and the Ethical Committee (transcript 23/14.11.2007). None of the patients had received nonsteroidal anti-inflammatory drugs or had recently been prescribed antibiotics for the last 3 months. Three biopsy specimens were collected from the antrum for the histology, culture, and rapid urease test (CLO test), as well as one biopsy specimen from the corpus and two biopsy specimens from the duodenum for histology.

Isolation and culture of *H. pylori*. Antral mucosa biopsy specimens collected from the greater curvature were aseptically placed in thioglycolate medium (Oxoid, Basingstoke, United Kingdom) and were processed for *H. pylori* isolation within 2 to 4 h after endoscopy. Specimens were vigorously vortexed with addition of sterile glass beads and cultured for up to 7 days on Chalgren-Wilkins agar plates containing antibiotics (vancomycin, 10 μg/ml; trimethoprim, 10 μg/ml; polymyxin B, 10^4 IU/liter; amphotericin B, 2 μg/ml; nalidixic acid, 10 μg/ml; bacitracin, 30 μg/ml; and fluorocytosine, 5 μg/ml) supplemented with 7% (vol/vol) horse blood and 1% (vol/vol) Vitox (Oxoid, Basingstoke, United Kingdom) under microaerophilic conditions (CampyPak Plus; Becton Dickinson, Cockeysville, MD) at 37°C. Plates were inspected daily for the presence of suspected colonies, which were initially screened for by colony morphology analysis and Gram staining and further verified by oxidase, catalase, and urease reactions. Culture sweeps, as well as individual colonies from each patient, were collected. *H. pylori* clonal relatedness within the same patient was routinely evaluated by randomly amplified polymorphic DNA (RAPD) PCR utilizing primers 1281, D14307, and D11344 (2). Multilocus sequence tagging (MLST) analysis with primers for the *atpA*, *efp*, *muuY*, *ppa*, *trpC*, *ureI*, *vacA*, and *yphC* housekeeping genes (19) was used to further characterize isogenic *H. pylori* strains expressing CagA with the divergent number of EPIYA motifs isolated from the same patient. In total, 105 *H. pylori* clinical isolates were collected and stored in brain heart infusion broth supplemented with 20% glycerol at −80°C until further analysis.

Characterization of diversity of EPIYA phosphorylation motifs. Characterization of the EPIYA phosphorylation motifs was accomplished utilizing our strategy as described before (34). Briefly, each clinical isolate was passed twice on Chalgren-Wilkins plates and total bacterial genomic DNA (optical density at 260 nm/optical density at 280 nm ≥ 1.800) was extracted using a DNeasy isolation kit provided by Qiagen AS (Oslo, Norway). The EPIYA-coding regions of the *cagA* gene were amplified (EPIYA PCR) utilizing primers cagA2530S (5′-GTTAAR AATRGTGTRAAYGG-3′, where R represents A or G and Y represents T or C) and cagA3000AS (5′-TTTAGCTTCTGATACCGC-3′), which recognize positions 582453 to 582977 with reference to the *H. pylori* 26695 genome. Amplicons ranging from 370 to 670 bp (±25 bp) were visualized by agarose gel electrophoresis and sequenced using a GenomeLab DTCS Quick Start sequencing kit (Beckman Coulter, Fullerton, CA) with a CEQ 8000 Beckman Coulter genetic analyzer. The number and type of EPIYA motifs were determined from the deduced peptide sequences following alignment by CLUSTAL W (European Bioinformatics Institute [http://www.ebi.ac.uk/Tools/clustalw2/index.html]). EPIYA PCR-negative cases were confirmed as true *cagA*-negative isolates by an empty-site-positive PCR assay as described before (1).

Detection of *vacA* gene diversity. VacA signal region isotyping was performed by PCR using the VA1-F and VA1-R primers, and middle region isotyping was carried out by multiplex PCR utilizing primers VA4-F, VA4-R, VA7-F, and VA7-R (7). Intermediate region genotypes were determined by PCR according to the instructions of Rhead et al. (39).

In vitro infection of gastric epithelial cells (AGS) with clinical isolates. Human gastric adenocarcinoma epithelial AGS cells (2×10^6 cells in 25-mm^2 flasks or 1×10^6 cells in six-well plates) cultured in F-12 Kaighn's medium (Gibco, Invitrogen, Ltd., Paisley, United Kingdom) containing 10% fetal bovine serum (Gibco) were infected with *H. pylori* clinical strains at a multiplicity of infection of 100 and incubated in a 5% CO_2 atmosphere. Total protein lysates and culture supernatants were collected at selected time points ranging from 1 to 48 h postinfection.

Expression and functional analysis of CagA phosphorylation. Total protein lysates from *H. pylori*-infected AGS epithelial cells were obtained in ice-cold lysis radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.2], 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, 2 mM L-dithiothreitol) containing protease and phosphatase inhibitor cocktails. Lysates with equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (7.5% polyacrylamide) and transferred onto polyvinylidene difluoride (Immobilon P; Millipore Corp., Bedford, MA) membranes. CagA expression was detected by Western blot analysis using an anti-CagA primary polyclonal antibody (Austral Biologicals, San Ramon, CA) followed by a secondary horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G polyclonal antibody (Jackson ImmunoResearch Europe, Ltd., Soham, Cambridgeshire, United Kingdom), detected by the ECL Plus

TABLE 1. CagA protein diversity with regard to EPIYA phosphorylation motifs in *H. pylori* isolates from symptomatic children

Strain group and EPIYA status[a]	No. (%) of strains
cagPAI negative	35 (33.3)
cagPAI defective	
ABC	9 (8.5)
ABCC	3 (2.8)
cagPAI functional	
AB	1 (1.0)
ABC	40 (38.1)
ABBC	1 (1.0)
ABCC	15 (14.3)
ACC	1 (1.0)
Total	105 (100.0)

[a] A, B, and C refer to the EPIYA A (EPIYAKVNKKK[A/T/V/S]GQ), EPIYA B (EPIY[A/T][Q/K]VAKKVNAKI), and EPIYA C (EPIYATIDDLG) motifs, respectively.

chemiluminescence detection system (Amersham, GE Healthcare UK, Ltd., Buckinghamshire, United Kingdom). CagA tyrosine phosphorylation was evaluated by detection of CagA expression following immunoprecipitation of total cell lysates with an anti-mouse monoclonal pY20 phospho-tyrosine antibody (BD Transduction Laboratories, Franklin Lakes, NJ).

Determination of IL-8 levels. Culture supernatants collected at selected time points over 48 h from in vitro *H. pylori*-infected AGS gastric epithelial cells (multiplicity of infection, 100) were centrifuged at 13,000 rpm, and the levels of IL-8 were determined by using a commercial enzyme-linked immunosorbent assay kit (Bender MedSystems GmbH, Vienna, Austria) according to the manufacturer's protocol.

Histological analysis. Biopsy specimens destined for histopathology evaluation were fixed in 10% neutral buffered formalin solution, processed for histology, and embedded in paraffin.

Several serial longitudinal 4-μm sections from each specimen were cut, two of them were stained with hematoxylin-eosin for evaluation of gastric inflammation, and one was analyzed with the May-Grünwald Giemsa method for assessment of *H. pylori* colonization. Bacterial density and pathology of gastric mucosa were assessed according to the updated Sydney System (18). Histopathological evaluation was performed by a histopathologist with no prior knowledge of the identity of the samples.

Statistical analysis. Statistical analysis with reference to potential associations between virulence factor variability and histopathological manifestations was conducted by using the SPSS package.

Nucleotide sequence accession numbers. The partial *cagA* nucleotide sequences generated in the present study were submitted to the GenBank/EMBL/DDBJ databases (accession numbers AM292556 and -7, AM292559 to -76, AM292579 to -95, AM295786, AM295789, and FM957544 to -62).

RESULTS

Patient demographics, clinical outcomes, and histopathologies. In total, 98 symptomatic children (49 male), aged 2 to 16 years (mean age, 10.7 ± 0.3 years), were included in the study. Symptoms included epigastric or abdominal pain (n = 73), pain coupled with vomiting (n = 10), or just vomiting (n = 15). Upon endoscopic evaluation, antral nodular gastritis was evident in 72 out of 98 patients (73.5%) and edema and erythema were evident in the corpus in 42 cases (42.7%), while in 6 children (5 male), duodenal ulcer was observed. Upon histological examination, all patients developed chronic active gastritis in the antrum, and 41 patients also developed it in the corpus. Neutrophil infiltration was assessed as mild (n = 9 patients), moderate (n = 80), or marked (n = 7). Mild (n = 5), moderate (n = 51), and marked (n = 40) lymphocytic infiltration was also observed. The presence of eosinophils was apparent in 43 patients (mild, n = 26; moderate, n = 17). *H. pylori* colonization was classified as absent (n = 9), mild (n = 10), moderate (n = 67), and marked (n = 10). Only one patient presented with atrophy and none with intestinal metaplasia. Lymphoid follicle formation was observed in 27 patients (18 male). Finally, no formation of ectopic gastric mucosa in the duodenum was observed in our sample population.

Determination of EPIYA diversity in CagA protein. Ninety-eight *H. pylori* clinical isolates were collected from our patients, and the bacterial DNA was subjected to EPIYA PCR for determination of EPIYA diversity. Thirty-five isolates (33.3%) were found to be negative in EPIYA PCR amplification (Table 1). These were all confirmed as true *cagPAI*-negative strains by empty-site positive PCR (1), which generates an amplicon in the absence of the whole pathogenicity island (data not shown). They were further verified by the absence of an immunospecific band in Western blot determination of CagA expression in individual protein lysates. In 56 isolates, single-band amplification by EPIYA PCR (Fig. 1A) and CagA protein expression (Fig. 1B) was observed. However, in the isolates from seven patients, we observed two amplified bands in the EPIYA-PCR (Fig. 1A). We verified by isolation and sequencing that these were *cagA*-specific gene sequences, indicating the presence of at least two infecting strains within the same patient. The expression of such dual CagA protein species was further verified by Western blot analysis of total protein lysates of AGS cells infected with the corresponding *H. pylori* strains (Fig. 1B), indicative of the simultaneous presence of two infecting strains expressing CagA protein with divergent

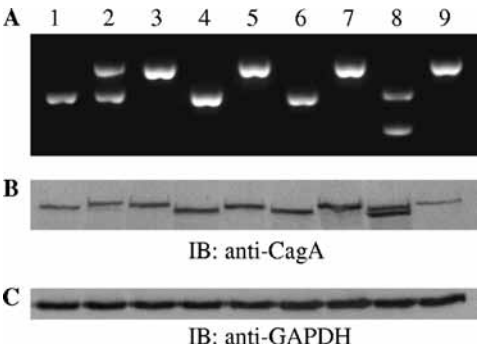

FIG. 1. CagA protein polymorphism with reference to EPIYA motifs. DNA from representative *H. pylori* clinical strains (lanes 1 to 9) was amplified by EPIYA PCR, and the PCR products were analyzed on a 1.5% agarose gel (A). Analysis of the expressed CagA proteins by immunoblotting (IB) (B) with anti-CagA rabbit polyclonal antibody, following in vitro infection of AGS gastric epithelial cells with the corresponding isolates shown in panel A. Note the presence of two amplified bands in samples 2 and 8 in panel A, denoting the presence of two distinct amplicons and the expression of the respective two different CagA protein species (B). The immunoblot in panel C depicts expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein as a total protein loading control for panel B.

ΞΕΝΟΓΛΩΣΣΕΣ ΔΗΜΟΣΙΕΥΣΕΙΣ ΕΛΛΗΝΩΝ ΕΡΕΥΝΗΤΩΝ

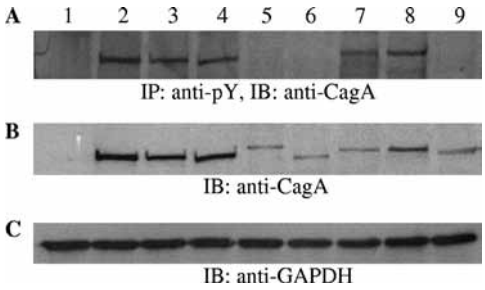

FIG. 2. Phosphorylation and expression of CagA protein following infection of gastric epithelial cells with representative *H. pylori* clinical isolates. CagA tyrosine phosphorylation (A) was evaluated by immunoblotting (IB) with anti-CagA rabbit polyclonal antibody following immunoprecipitation (IP) of total cell lysates with an anti-mouse monoclonal pY20 phospho-tyrosine antibody. CagA expression (B) was determined by immunoblotting with anti-CagA rabbit polyclonal antibody on total cell lysates. Lane 1, *cagPAI*-negative isolate. Lanes 2 to 4 and 7 and 8, isolates with functional *cagPAI* and CagA protein with three (ABC) and four (ABCC) EPIYA motifs, respectively. Lanes 5, 6, and 9, *cagA*-positive isolates with defective *cagPAI* resulting in the absence of CagA protein phosphorylation. The immunoblot in panel C depicts expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein as a total protein loading control for panel B. Anti-CagA rabbit polyclonal antibody and anti-GAPDH mouse monoclonal antibody were utilized at 1:4,000 and 1:10,000 dilutions, respectively, in the presence of 5% nonfat dry milk. Protein separation by SDS-polyacrylamide gel electrophoresis was realized using 6% polyacrylamide gels.

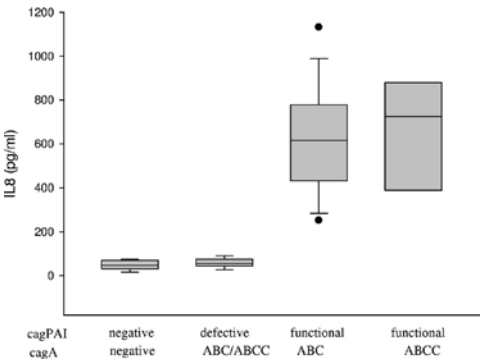

FIG. 3. Levels of IL-8 secreted by gastric epithelial cells following 48-hour in vitro infection with *H. pylori* clinical isolates. *H. pylori* strains are classified as *cagPAI* negative, *cagPAI* defective, and *cagPAI* functional, with the CagA protein harboring EPIYA motifs in the ABC and ABCC combinations.

numbers of EPIYA motifs. We proceeded to separate these pairs of isolates by following the methodology described in our earlier publication (34), bringing the total number of strains to 105. More specifically, we successfully separated those subclones by limiting dilution, *H. pylori* colony selection, and screening of individual colonies for a single PCR amplicon by our EPIYA PCR. RAPD PCR analysis on genomic DNA derived from the isolated pairs of strains revealed identical profiles (see Fig. S1 in the supplemental material), an indication that these strains were clonally closely related. The subclones were further subjected to MLST analysis, which afforded identical sequences for all seven genes (data not shown), suggesting that the isolated clones were isogenic. For six out of seven patients, sequence analysis of the EPIYA PCR amplicons revealed the simultaneous presence of CagA protein species with either three or four EPIYA domains (ABC/ABCC combination). Upon comparison, sequences were found to be identical on a nucleotide basis outside the 102-bp sequence repeat coding for the 34-amino-acid peptide segment containing the additional EPIYA C motif. The remaining isolate contained two subclones expressing CagA with either an AB or an ABC combination of EPIYA motifs.

The nucleotide sequences of the EPIYA-PCR amplicons from all 105 *H. pylori* isolates were aligned, and the deduced peptide sequences revealed the presence of EPIYA motifs, namely, EPIYA A (GLKN[ST]EPIYAKVNKKK), EPIYA B (Q[V/A]ASPEPIY[A/T]QVAKKV), and EPIYA C (RS[V/A]SPEPIYATIDDLG). The ST dipeptide preceding the EPIYA A motif was observed in only 15 patients. The majority of isolates contained alanine (A) ($n = 46$) within the EPIYA B motif; however, equal distributions of valine and alanine were observed for EPIYA C. No significant variations in the individual peptide sequences surrounding the EPIYA motifs were observed in our sample from children and those already published for adult populations in Greece (34). In all, 49 out of 105 (46.7%) isolates were found to possess three EPIYA domains in the ABC conformation and 18 (17.1%) isolates four domains in the ABCC conformation (Table 1). Rare cases of CagA proteins with EPIYA domains in combinations such as ABBC ($n = 1$) and ACC ($n = 1$) were also observed, whereas no cases with more than two EPIYA C repeats were detected. For the *cagA*-positive isolates, *cagPAI* functionality was assessed by determination of CagA protein phosphorylation (Fig. 2) and levels of secreted IL-8 in the supernatant (Fig. 3), following incubation of *H. pylori* strains with AGS gastric epithelial cells. In this way, we identified 12 isolates with defective *cagPAI* within our *cagA*-positive population, 9 with the ABC and 3 with the ABCC combination of EPIYA (Table 1).

Classification of strains according to *cagA* EPIYA diversity and *cagPAI* status. In view of the critical role of *cagPAI*-encoded proteins in successful translocation of CagA protein inside the gastric epithelial cell and the putative role of phosphorylated EPIYA C motifs in increased deregulation of SH2 domain-containing protein-tyrosine phosphatase-2 (SHP2 phosphatase) (24), we grouped our isolates according to the presence of phosphorylated CagA protein as well as the number of EPIYA C motifs. Consequently, *cagA*-negative strains, as well isolates for which we could not detect phosphorylated CagA, were grouped together ($n = 48$ isolates) (Tables 2, 3, and 4). In this group, a *cagA*-positive strain without EPIYA C motifs (AB) was also included. Forty-one isolates bearing three EPIYA motifs in an ABC combination and one isolate with the ABBC combination of EPIYA motifs, all with functional *cagPAI*, were classified in the group with one EPIYA C motif. Accordingly, 16 isolates with CagA bearing four EPIYA motifs (ABCC) as well as 1 isolate with the ACC combination and

TABLE 2. CagA protein diversity with regard to EPIYA-*C* motifs and *vacA* signal, intermediate, and middle region isotypes

VacA isotype	No. of isolates with indicated no. of EPIYA C repeats[a] in CagA protein (%)		
	Zero	One	Two
s1			
i1/m1	3 (2.9)	11 (10.5)	6 (5.7)
i1/m2	4 (3.8)	3 (2.9)	2 (1.9)
i1-i2/m1	2 (1.9)	7 (6.7)	0
i1-i2/m2	6 (5.7)	7 (6.7)	2 (1.9)
i2/m1	0	1 (1.0)	0
i2/m2	4 (3.8)	9 (8.6)	4 (3.8)
s2			
i1/m2	0	1 (1.0)	1 (1.0)
i1-i2/m2	10 (9.5)	2 (1.9)	1 (1.0)
i2/m2	19 (18.1)	0	0

[a] The presence of the characteristic EPIYA C motif (EPIYATIDDLG) is indicated. "Zero" refers to the absence of tyrosine-phosphorylated CagA.

functional *cagPAI* were classified in the group with two EPIYA C motifs. No correlation was observed between EPIYA C diversity and gender of host (Pearson's $\chi^2 = 0.787$; $P = 0.675$). Finally, no correlation was observed between EPIYA C status and reason for referral (Pearson's $\chi^2 = 2.773$; $P = 0.597$), as the overwhelming majority of our patients were referred for recurrent abdominal pain.

Determination of *vacA* diversity and correlation with *cagA* genotype. We characterized all isolates according to the *vacA* signal, intermediate, and middle region isotypes (Table 2). In the absence of functional EPIYA C motifs (Table 2), the majority of isolates (41 out of 48) were found to possess presumed nonvacuolation (s2/i2/m2 [$n = 19$; 18.1%] and s1/i2/m2 [$n = 4$; 3.8%]) or low-vacuolation (s1/i1-i2/m1 or m2 [$n = 8$; 7.6%] and s2/i1-i2/m2 [$n = 10$; 9.5%]) isotypes, whereas only 7 (6.7%) isolates were of high-vacuolation (s1/i1/m1 or m2) isotypes. No isolates bearing the s2/m1 isotype, irrespective of intermediate region type, were observed. In the overwhelming majority of CagA-positive strains with functional *cagPAI*, the presence of EPIYA C motifs was positively correlated (Pearson chi-square test; $P < 0.001$) with high-vacuolation (s1/i1/m1 or m2 [$n = 22$; 21.0%]) or lower-vacuolation (s1/i2 or i1-i2/m1 or m2 [$n = 30$; 28.8%]) isotypes. Only five (4.9%) among the *cagA*-positive isolates were found to be of nonvacuolation (s2/i1 or i2 or i1-i2/m2) isotypes. A positive correlation between presence of high-vacuolation isotypes and increasing age was observed (Pearson chi-square test; $P = 0.026$) (Table 3). Interestingly, in 12 out of 17 cases where two EPIYA C motifs were present (including cases of mixed ABC/ABCC infection), the patients were over 10 years old (Table 3). Therefore, a positive correlation between higher number of EPIYA C motifs in the CagA protein and increasing age may also exist, but analyses of more cases are required for determination of this.

Association of EPIYA diversity in CagA protein and *vacA* isotypes with histopathology in the antrum. To facilitate statistical analysis, isolates were classified according to (i) the numbers of EPIYA C motifs in the groups, namely none, one, or two, and (ii) the levels of vacuolating potential, namely, high for s1/i1/m1-or-m2 isotypes, low for s1-or-s2/i1-i2 isotypes, and none for s1-or-s2/i2 isotypes, irrespective of the middle region isotype. This classification was utilized for the assessment of possible associations with the corresponding histological manifestations in the 91 patients where infection by a single *H. pylori* strain was detected (Table 4). No correlation was observed between number of repeating EPIYA motifs and either chronic gastritis activity, chronic inflammatory infiltration, or levels of *H. pylori* colonization in the antrum in the children in our study ($P = 0.571$, $P = 0.193$, and $P = 0.074$, respectively) (Table 4). Also, no correlation with grade of eosinophil infiltration ($P = 0.957$) or presence of lymphoid follicle formation ($P = 0.912$) was observed (Table 5). Furthermore, with reference to the *vacA* genotype, we did not observe any correlation with chronic gastritis activity, chronic inflammatory infiltration, or levels of *H. pylori* colonization in the antrum ($P = 0.054$, $P = 0.499$, and $P = 0.230$, respectively) (Table 4) or with eosinophil infiltration or formation of lymphoid follicles ($P = 0.341$ and $P = 0.283$, respectively) (Table 5). Interestingly, the only significant correlation was the one between *vacA* genotype and presence of nodular gastritis (Pearson chi-square test; $P = 0.034$) (Table 6), an endoscopic observation quite common in cases of *H. pylori*-associated gastritis in children. All correlations between EPIYA C status and different *vacA* isotypes and clinical and histopathological manifestations in the antrum were found to be nonsignificant. Collectively, these results suggest that the number of repeating EPIYA C motifs in CagA protein, coupled with the distinct *vacA* isotypes, does not seem to correlate with severity of gastric inflammation in *H. pylori*-infected children.

Induction of IL-8 levels and relation to virulence factor variability. In order to evaluate whether potential differences in IL-8 level depended upon EPIYA diversity, we infected AGS cells in vitro over a 48-h period with *H. pylori* isolates (i) without *cagPAI* ($n = 18$), (ii) with *cagPAI* present but defective ($n = 10$), and (iii) with CagA positivity and functional *cagPAI* and EPIYA motifs in the ABC ($n = 19$) or ABCC ($n = 7$) combination and determined the levels of IL-8 protein secreted in the supernatant. No significant difference in IL-8

TABLE 3. VacA and CagA diversity in relation to patient age

Patient age	No. of isolates with indicated vacuolation level[a] (%)			No. of isolates with indicated no. of EPIYA C repeats[b] in CagA protein (%)		
	None	Low	High	Zero	One	Two
≤10 yr	19 (19.4)	16 (16.3)	5 (5.1)	20 (20.4)	15 (15.3)	5 (5.1)
>10 yr	17 (17.3)	18 (18.4)	23 (23.5)	26 (26.5)	20 (20.4)	12 (12.2)

[a] VacA isotypes were classified into high-vacuolation (s1/i1), low-vacuolation (s1 or s2/i1-i2), and nonvacuolation (s1 or s2/i2) groups, irrespective of middle region status.
[b] The presence of the characteristic EPIYA C motif (EPIYATIDDLG) is indicated. "Zero" refers to the absence of tyrosine-phosphorylated CagA.

ΞΕΝΟΓΛΩΣΣΕΣ ΔΗΜΟΣΙΕΥΣΕΙΣ ΕΛΛΗΝΩΝ ΕΡΕΥΝΗΤΩΝ

TABLE 4. CagA and VacA diversity among *H. pylori* isolates in relation to colonization levels and associated gastritis in the antrum

VacA isotype and no. of EPIYA C repeats[a]	No. of patients[b] with:									
	H. pylori colonization				Chronic gastritis activity			Chronic inflammatory infiltration		
	Normal	Mild	Moderate	Marked	Mild	Moderate	Marked	Mild	Moderate	Marked
High vacuolation										
0	1	1	4	0	0	6	0	0	4	2
1	1	1	7	3	0	10	2	0	5	7
2	1	3	2	0	0	6	0	0	6	0
Low vacuolation										
0	0	3	13	1	2	15	0	0	10	7
1	0	0	11	2	0	13	0	0	8	5
2	0	0	0	0	0	0	0	0	0	0
No vacuolation										
0	2	2	16	3	4	17	2	3	12	8
1	1	0	8	0	2	6	1	1	1	7
2	1	0	2	0	0	2	1	0	0	3

[a] VacA isotypes were classified into high-vacuolation (s1/i1), low-vacuolation (s1 or s2/i1-i2), and no-vacuolation (s1 or s2/i2) groups, irrespective of middle region status. For each isotype, the presence of the characteristic EPIYA C motif (EPIYATIDDLG) is indicated. "0" refers to the absence of tyrosine-phosphorylated CagA.
[b] The severity levels (normal, mild, moderate, and marked) are defined in reference 18.

levels was observed between *cagA*-positive isolates with one and two EPIYA C motifs in the CagA protein (mean levels of 615.5 ± 51.6 pg/ml and 724.9 ± 89.7 pg/ml, respectively) (Fig. 3). As expected CagA-negative isolates lacking functional *cagPAI* induced only basal IL-8 levels ($n = 12$ isolates; 48.4 ± 7.7 pg/ml). Within the CagA-positive population tested, 62.8% ($n = 22$) of the isolates were positive for the presence of phosphorylated CagA protein and induction of IL-8 secretion. Finally, we sought to determine the potential difference in level of secreted IL-8 between the individual isogenic subclones expressing CagA protein with variable numbers of EPIYA C repeats isolated from the same patient. AGS cells were infected over a 48-h period with the individual *H. pylori* isogenic subclones expressing CagA with EPIYA repeats in the AB or ABC combination (GenBank accession numbers AM292594 and AM292593) and a second pair of subclones with the ABC or ABCC combination (GenBank accession numbers AM292577 and AM292578). No differences in level of secreted IL-8 were observed between the two isogenic subclones expressing CagA with EPIYA in the AB or ABC combination (1,331.5 ± 129.0 versus 1,319.0 ± 128.2 pg/ml) or between the two subclones with the ABC or ABCC combination (1,384.3 ± 101.6 pg/ml versus 1,242.7 ± 112.2 pg/ml).

Consequently, no correlation between level of secreted IL-8 and EPIYA combination was observed. Also, no correlation was observed between IL-8 level and chronic gastritis activity, chronic inflammatory infiltration, or level of *H. pylori* colonization in the antrum (data not shown).

TABLE 5. CagA and VacA diversity among *H. pylori* isolates in relation to eosinophil infiltration and lymph node hyperplasia in the antrum

VacA isotype and no. of EPIYA C repeats[a]	No. of patients with:				
	Eosinophil infiltration[b]			Lymph node hyperplasia	
	Mild	Moderate	Marked	No	Yes
High vacuolation					
0	2	2	2	5	1
1	6	5	1	8	4
2	2	2	2	6	0
Low vacuolation					
0	9	6	2	14	4
1	7	4	2	10	3
2	0	0	0	0	0
No vacuolation					
0	14	5	4	15	8
1	7	0	2	6	3
2	2	1	0	1	2

[a] VacA isotypes were classified into high-vacuolation (s1/i1), low-vacuolation (s1 or s2/i1-i2), and nonvacuolation (s1 or s2/i2) groups, irrespective of middle region status. For each isotype, the presence of the characteristic EPIYA C motif (EPIYATIDDLG) is indicated. "0" refers to the absence of tyrosine-phosphorylated CagA.
[b] The severity levels (mild, moderate, and marked) are defined in reference 18.

DISCUSSION

In the present study, we have examined the potential correlation between *H. pylori* virulence factor diversity and endoscopic and histopathologic manifestations in symptomatic children. With relation to the CagA protein, EPIYA phosphorylation motif diversity has been suggested to be a major contributor to *H. pylori* pathogenesis in adults (12). From a mechanistic point of view, CagA has been suggested to interact in a

TABLE 6. VacA isotypes and endoscopic observation of nodular gastritis

Nodular gastritis status	No. (%) of isolates with indicated vacuolation level[a]		
	High	Low	None
Absent	9 (9.9)	3 (3.3)	12 (13.2)
Present	16 (17.6)	28 (30.8)	23 (25.3)

[a] VacA isotypes were classified into high-vacuolation (s1/i1), low-vacuolation (s1 or s2/i1-i2), and nonvacuolation (s1 or s2/i2) groups, irrespective of middle region status.

phosphorylation-dependent manner with and perturb the normal activity of the tyrosine phosphatase SHP2 (26), the C-terminal Src kinase Crk (47), and the Crk adaptor proteins (45). Deregulation of SHP2 and its downstream effector focal adhesion kinase (48) was suggested to be mediated via the EPIYA C motif and, for the C-terminal Src kinase (Csk), via the EPIYA A or EPIYA B motif (29). In this respect, careful characterization of clinical isolates with reference to their ability to express functional CagA and determination of EPIYA diversity could provide useful predictive tools for *H. pylori* pathogenesis. Nearly 47% of *cagA*-positive isolates from the population of children analyzed in this study harbored three EPIYA motifs in the ABC combination, and only 17% harbored four EPIYA domains in the ABCC combination. Furthermore, among *cagA*-positive strains, about 19% (12 out of 63) were found to lack the capacity to express functional CagA protein and induce IL-8 secretion following infection of gastric epithelial cells. No significant variations in the individual peptide sequences surrounding the EPIYA motifs were observed in our sample from children and those already published for adult patients in Greece (34). However, although EPIYA phosphorylation motifs of the CagA protein in *H. pylori* strains isolated from Greek children do not present structural differences compared to those prevailing in adults, some subtle variations were noted. We observed a trend toward higher prevalence of *cagA* negativity in strains isolated from children than in those isolated from adults, an observation that has already been reported for populations with much higher prevalences of *H. pylori* infection (40). The higher prevalences of *cagA*- and *cagPAI*-negative *H. pylori* strains among children may reflect an evolutionary modification permitting successful colonization during transmission. Indeed, studies involving murine models (37) have shown that *H. pylori* strains in which *cagPAI* is absent or in which there is loss of functions required for activation of NF-κB exhibit a selective advantage for colonization of the murine host. Therefore, there may be a selective pressure for colonizing *H. pylori* strains to lose such features in vivo. Furthermore, we observed a positive correlation between number of EPIYA C motifs in CagA and age; in 12 out of 17 cases where two EPIYA C motifs were present (including mixed ABC/ABCC), the patients were over 10 years old. In contrast to what was found for the *H. pylori* strains isolated from adults (34), we did not detect any CagA-positive isolates harboring more than two EPIYA C repeats in children, and this may be related to reduced rates of survival of such strains in acidic conditions (49). As far as the contribution of CagA to *H. pylori* pathogenesis is concerned, this fact may offer a good explanation for the reduced severity levels of clinical and pathological manifestations associated with *H. pylori* infection in children compared to the levels observed in adults. Interestingly, we detected simultaneously present microevolving strains in the antrum with identical RAPD and MLST profiles, expressing CagA proteins with variable numbers of EPIYA motifs, including one pair with AB/ABC combinations and six pairs with ABC/ABCC combinations of EPIYA motifs in CagA. This may suggest that children when first acquiring the infection may be colonized by multiple *H. pylori* variants which, over time, through selection influenced by host genetics and bacterial factors, result in various *H. pylori* genotypes that predominate in adulthood. Similar results suggesting that, after infection in childhood, individual strains will undergo evolutionary changes during the course of infection have been reported before (33).

With relation to *vacA* status, we have observed an association between *cagA*-negative genotype and nonvacuolation (s2/i1 or i2/m2) *vacA* isotypes. Also, *cagA*-positive strains with one or two EPIYA C repeats were mostly associated with high-vacuolation (s1/i1/m1) or lower-vacuolation (s1 or s2/i1-i2) isotypes, suggesting that *cagA* and *vacA* genotypes may not be regarded as independent variables in *H. pylori* pathogenesis. Indeed, there is increasing evidence that a functional association between *vacA* and *cagA* virulence factors may exist (4). In line with this observation, isolates from our pediatric population with high vacuolation potential and higher numbers of EPIYA C motifs in the CagA protein were more prevalent among children over 10 years old.

Induction of IL-8 secretion following *H. pylori* infection of gastric epithelial cells is largely regarded to be a *cagPAI*-dependent phenomenon (43). However, there is mounting evidence that translocated CagA protein may also contribute to IL-8 induction through a Ras/Raf/Mek/extracellular signal-regulated kinase/NF-κB signaling pathway (15). We determined levels of secreted IL-8 in the supernatant of gastric epithelial cells, infected in vitro with a variety of strains, including *cagA*-negative as well as *cagA*-positive *H. pylori* isolates bearing one or two EPIYA C repeats in the CagA protein, with or without functional *cagPAI*. In this context, apart from IL-8 secretion, *cag* pathogenicity island functionality was further established by simultaneously screening for the presence of phosphorylated CagA protein in protein lysates from *H. pylori*-infected AGS cells. We observed no correlation between number of repeating EPIYA C motifs and level of proinflammatory IL-8 secreted by gastric epithelial cells. IL-8 levels were markedly different between patients, irrespective of the number of EPIYA motifs in CagA, an observation also reported by others (30, 38). Furthermore, to account for unknown variability between the different clinical strains, we utilized pairs of isogenic strains with variable numbers of EPIYA C, one of which totally lacked EPIYA C motifs. We observed no differences in level of secreted IL-8 between those isogenes with zero, one, or two EPIYA C repeats, suggesting that the contribution of the number of EPIYA C repeats in the CagA protein to IL-8 secretion may be marginal.

Although our analysis was considerably thorough, in order to characterize strains with reference to the functional status of CagA and VacA virulence factors and not just the genotypic status, no correlation between virulence factor diversity and clinical symptoms was observed, as the overwhelming majority of our patients were referred for endoscopy with symptoms of recurrent abdominal pain. Also, no correlation between virulence factor diversity and developing histopathology in the antrum and the fundus in children was observed. Similar conclusions with reference to the presence of CagA and the signal and middle region genotypic variation of VacA have been reported before in a number of studies involving *H. pylori*-infected children (9, 21, 33), but never associating functional characteristics which contribute to the pathogenic role of CagA, *cagPAI* functionality, or the more recently reported intermediate region VacA isotypes. Interestingly, the only positive, yet weak, correlation was the one observed between vac-

uolating *vacA* genotypes and presence of nodular gastritis, an endoscopic observation quite common in cases of *H. pylori*-associated gastritis in children. Such positive correlation was not observed in earlier studies involving *H. pylori* bacterial genotypes and endoscopic observations in children and may warrant further verification. *H. pylori* prevalence in biopsied symptomatic children in Greece is estimated around 14% on the basis of detection by culture or histology (E. Roma-Giannikou, unpublished data), which is in line with what is observed in other northern and western European countries (46). The lack of correlation between *H. pylori* virulence factor diversity and severity of histopathology in symptomatic children may correlate with differences in the course of infection, possibly with correlations becoming stronger as the duration of infection increases and potential increments in the number of repeating EPIYA motifs in CagA occur throughout adulthood. This is further supported by our observations that potentially more virulent forms of *H. pylori* were found to be present in older children and that CagA species with three or even four EPIYA C repeats are exclusively observed in strains isolated from adults and not from children.

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There are no potential conflicts of interest.

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Early-Stage Gastric MALT Lymphoma: Is It a Truly Localized Disease?

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Target audience: Physicians who wish to advance their current knowledge of clinical cancer medicine in lymphoma oncology.

LEARNING OBJECTIVES

After completing this course, the reader should be able to:

1. In your patients with gastric MALT lymphoma (GML), determine accurate staging and formulate appropriate treatment strategies.
2. Assess early stage GML patients who should be closely monitored for early intervention and manage treatment plans.
3. Design further studies with different modality treatments to explore the impact of occult blood disease on patients' outcomes.

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ABSTRACT

Early-stage gastric mucosa-associated lymphoid tissue lymphoma (GML) is considered a localized disease with an indolent course. Circulating malignant cells have been detected in other early-stage indolent lymphomas by molecular methods. We investigated the incidence of occult blood disease in early-stage GML patients, its impact on clinical outcome, and the similarity between blood and gastric lymphocytic clones. Sixty-two patients with local-

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ized GML were included in the study; 51 of them had *Helicobacter pylori* infection. Monoclonality was investigated by leader polymerase chain reaction. Sequencing was performed for the immunoglobulin variable gene (*VH*) analysis. Blood involvement was absent in all patients by conventional staging methods. In the whole group of 62 patients, the incidence of blood *IgH* rearrangement was 45%, and this did not correlate with baseline patient characteristics. The monoclonal blood and gastric products of five patients were sequenced and compared with each other. Clonal identity was evident in four of five patients. The *VH3* gene was the most frequently used, both in the blood and in the stomach. Early-stage GML is not a truly localized disease because half the patients had a circulating clone, probably identical to the gastric one. The clinical significance of occult blood disease and the potential appropriate intervention need to be further investigated. *The Oncologist* 2009;14:148–154

INTRODUCTION

Mucosa-associated lymphoid tissue (MALT) lymphomas represent 7%–8% of B-cell lymphomas. Gastric MALT lymphoma (GML) comprises almost 50% of all MALT lymphomas. GML remains localized for a long period of time and is considered an indolent disease, although transformation to large-cell lymphoma may occur [1, 2]. *Helicobacter pylori* (Hp) infection is associated with GML, as demonstrated by numerous studies [3–5]. First-line treatment for early-stage GML is the eradication of Hp with antibiotics, producing complete lymphoma remission in a large proportion of patients [6, 7]. However, varying percentages (30%–65%) of patients fail to respond to antibiotics, especially those with t(11,18)(q21;q21) translocation [8–10]. For patients who are Hp^- and those who fail anti-Hp antibiotics, there is no standard treatment.

As already pointed out, the majority of GML patients present with early-stage disease. However, it has been suggested that early-stage GML is not a truly localized disease, as is the case with localized follicular lymphoma, for which occult blood involvement has been documented by molecular methods [11–13]. The aim of the present study was to investigate whether a clonal lymphocytic population circulates in the blood of early-stage GML patients, to compare it with the gastric malignant clone, and to explore whether it has an impact on patient clinical status and outcome.

PATIENTS AND METHODS

Patient Characteristics

Of the 80 patients with GML referred to our department during the last decade, 62 had localized GML (stage I or II_1) and were included in the present study. Fifty-nine patients had stage I (95%) and three patients had stage II_1 disease according to the modified Blackledge staging system [14, 15]. There were 36 men and 26 women, with a median age of 57 years (range, 33–86). Epigastric pain was the most frequent symptom (46%), followed by bleeding (19%) and belching (12.7%). GML was found during routine investigation in three (4.8%) patients. Hp was found to be positive in 51 of 62 patients (82%).

Diagnosis and Staging

The diagnosis was documented by histological and immunohistochemical studies after gastrointestinal endoscopy in all but eight patients, who were diagnosed after surgery. The histological diagnosis was based on Wotherspoon's histological index [7]. For the documentation of Hp infection, modified Giemsa staining on histological sections was used.

Staging procedures included history and physical examination, CBC with differential, biochemical profile, computed tomography scans of the thorax and abdomen, as well as bone marrow aspiration plus biopsy. For the exclusion of lymphomatous blood involvement, immunophenotypic analysis by flow cytometry was performed, using the following monoclonal antibodies (Becton-Dickinson, San Jose, CA): anti-CD20, anti-CD19, anti-CD5, anti-HLA-DR, anti-CD38, anti-CD23, anti-CD4, anti-CD8, anti-CD45, and anti-CD14 [16]. Staging was evaluated using the modified Blackledge system [14]. In brief, stage I includes cases with a single lesion or multiple noncontiguous lesions in the stomach without serosal penetration, whereas stage II_1 refers to cases with gastric involvement and local lymph node enlargement.

Polymerase Chain Reaction Methodology

To examine monoclonal B-cell populations in blood and gastric biopsies, analysis of *IgH* gene rearrangement by leader polymerase chain reaction (PCR) was performed [17–19]. DNA was extracted from blood mononuclear cells according to the phenol-chloroform protocol and from paraffin-embedded, formalin-fixed gastric biopsies after deparaffinization with xylene, ethanol precipitation, and proteinase K digestion, followed by the phenol-chloroform method. DNA was amplified using the MJR System with 20 pM of each of the *VH* family leader primers corresponding to the *VH* family sequences and two consensus downstream *JHA* and *JHB* primers corresponding to consensus sequences of the J region (Table 1). The PCR contained 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0), 1.5 mM $MgCl_2$, 200 mM of each deoxynucleotide triphosphate, and 2.5U Taq platinum (Invitrogen, Carlsbad, CA) at a final volume of 50 μl. Thirty-five cycles of amplification

Table 1. Primers for the IgH rearrangement by polymerase chain reaction

Gene	Primer
VH1 + 7L	5′-ATGGACTGGACCTGGAGG
VH2L	5′-CACGACTCCTGCTGCTGACCA
VH3L	5′-GCTGGGTTTTCCTTGTTGC
VH3bL	5′-ATGGAGTTTGGGAGCTGAGCTG
VH4L	5′-GCTCCCAGATGGGGTCCTG
VH5L	5′-CTCCTCCTGGCTGTTCTCC
VH6L	5′-CTGTCTCCTTCCTCATCTCC
JHA	5′-GAGGAGACGGTGACCAGGGT
JHB	5′-GAGGAGACAGTGACCAGGGT

were performed under the following conditions: denaturation at 94°C for 45 seconds, annealing at 65°C for 45 seconds, and extension at 72°C for 45 seconds. The reaction was completed with a final extension at 72°C for 10 minutes. The PCR products were analyzed on a 3% agarose gel and visualized under UV light after staining with ethidium bromide.

Cloning and Sequencing

The PCR products of the expected size were purified by affinity columns (NucleoSpin Extract, Macherey-Nagel, Germany). The recovered DNA was ligated into the PCR 4-Topo vector according to the manufacturer's instructions (Topo TA Cloning Kit, Invitrogen, Carlsbad, CA). Ten colonies for each sample were randomly picked and grown overnight in 3 ml LB medium. Recombinant plasmids were purified by Qiagen miniprep columns (Qiagen, Germantown, Maryland) and selected by restriction analysis using EcoR1 (Roche Diagnostics GmbH, Mannheim, Germany). The plasmid DNA was sequenced in an automated sequencer (ABI capillary sequencer, MWG Biotech, Ebersberg, Germany) [20]. Sequences obtained from each blood and gastric clone were aligned with germline sequences in the IMGT/V-QUEST directory (http://imgt.cines.fr). The similarity of the *VH* genes to the closest germline sequences was defined as a percentage in the range of 98%–100% for the unmutated genes and <98% for the mutated ones [21]. Replacement mutations (leading to amino acid change) and silent mutations (no amino acid change) were calculated in framework regions and in complementarity determining regions (*CDR*s) in all mutated immunoglobulin sequences.

Statistical Analysis

Statistical analysis was performed using the χ^2 test and Student's *t*-test. A probability value of $p<0.05$ was considered statistically significant. Progression-free survival (PFS) and overall survival (OS) were calculated according to the method of Kaplan and Meier [22]. The possibility of antigen selection in the mutated sequences was calculated using the multinomial distribution model by Lossos et al. [23].

Treatment and Follow-Up

All patients with Hp infection received antibiotics as the sole initial treatment [24, 25]. Patients underwent endoscopy 2 months after the antimicrobial therapy for the evaluation of Hp eradication and lymphoma response. Thereafter, patients were followed by endoscopy with multiple biopsies and imaging studies every 4–6 months for the first 2 years and once a year thereafter up to 5 years. Patients with no response or progressive disease after 4–6 months from antibiotic treatment and patients without Hp infection were treated with other modalities.

Response Criteria

For the definition of histological response, the Wotherspoon index [7] and the criteria published by Neubauer et al. [26] were used. Complete response (CR) was defined as the resolution of all disease-related symptoms, the disappearance of endoscopic lesions, and the accomplishment of histological remission according to Neubauer et al. [26], or a score <3 according to the Wotherspoon index.

RESULTS

Molecular Study by PCR

In all patients, a blood immunophenotypic analysis did not reveal monoclonal B cells. Gastric DNA was available for analysis in 21 of 62 patients, whereas blood DNA was available in all. Monoclonality was documented by PCR in 20 of 21 gastric biopsies (95%). Rearrangement of *IgH* of the same size was detected in the blood of 28 of 62 (45%) patients. The single patient who was molecularly negative in the stomach was negative in the blood as well.

Furthermore, the presence of blood *IgH* monoclonality was correlated with Hp infection and clinical stage. Among the Hp^+ patients, 26 of 54 (48%) were found with blood *IgH* rearrangement, whereas only two of eight (25%) Hp^- patients were *IgH* rearranged in the blood. However this difference was not statistically significant. Twenty-five (42%) patients with stage I had occult blood involvement as assessed by PCR, versus three (100%) of the stage II_1 patients, but again this difference did not reach statistical significance.

Sequencing Analysis of the VH Genes

Among the 20 patients with available blood and gastric material, five with positive *IgH* rearrangement in both compartments were randomly picked for sequencing analysis. The *VH* gene usage as well as the clonal identity to the germline se-

quences in blood and gastric clones for each of the five patients are shown in Table 2. The most commonly represented *VH* gene both in the blood and stomach was *VH3* (in four of five patients) whereas the *VH2* family was present in one patient. In four cases (patients 1–4), the monoclonal sequences detected in the stomach were the same as the sequences detected in the blood. In patient #5, two clonal products were evident in the blood by the leader PCR. Sequencing analysis detected only one productive *Ig* sequence, which was different from the gastric clone. Three patients (patients 2, 3, and 5) had mutated genes and two had unmutated genes. In patients 2 and 3, the gastric clones displayed a higher number of mutations than the corresponding blood clones. The ratio of replacement to silent mutations is shown in Table 2.

Response to Treatment and Outcome

Among 51 patients with Hp infection, 50 received antibiotics as the sole initial therapy. Eradication of Hp was successful in all patients. Ten of 50 (20%) patients had a complete lymphoma response. Patients who did not respond to antibiotics, as well as those who were Hp^- were treated with other modalities, as shown in Table 3. CR to anti-Hp treatment did not correlate with the presence of blood clone. Thus 16% of the blood IgH^+ patients achieved a CR, versus 21% of the blood IgH^- patients.

During a median follow-up of 67 months (range, 9–179), 15 patients relapsed/progressed—14 in the stomach and one in the liver and small intestine. The 5-year PFS rate for all patients was 75%. The PFS rate for blood IgH^+ patients was 70% and the PFS rate was 77% for IgH^- patients ($p = .27$). The 5-year OS rate for all patients was 97%—92% for blood IgH^+ and 100% for blood IgH^- patients ($p = .12$). In addition, the presence of blood *IgH* rearrangement did not correlate with PFS and OS, in either Hp^+ or Hp^- patients. Furthermore, the presence of blood clonality did not influence patient outcome or the pattern of relapse for patients who received local treatment. In detail, three local relapses were observed among eight patients who were treated with surgery or radiotherapy. Two patients were IgH^- and one was IgH^+ in the blood.

Discussion

Marginal B-cell lymphomas of the MALT type are considered indolent diseases with a long survival time [27–29]. Half of them involve the stomach and are localized at diagnosis [1, 30]. It is now known that chronic antigenic stimulation resulting from Hp infection plays a central role in the pathogenesis of GML [5, 3, 31]. The accurate staging of a GML is still under investigation [32]. Using the modified Blackledge system, most GMLs are found at early stages, stage I and II_1 [33, 15]. It has been proven that occult blood disease may be present using sensitive methods in localized lymphoproliferative diseases such as early-stage follicular lymphoma [12]. In addition, patients with follicular lymphoma in complete remission with conventional staging often have a circulating clone in the blood, demonstrated by PCR [13, 34]. MALT lymphoma is considered a truly localized disease at stages I and II_1.

Based on the above observations, we investigated whether a monoclonal population is present in the blood of patients with early-stage GML without any evidence of blood involvement by morphology and immunophenotyping. We studied 62 stage I and II_1 patients with histologically proven GML. Leader PCR was found to have an excellent sensitivity (95%) in the detection of the lymphomatous clone in the stomach [17–19]. *IgH* rearrangement in gastric biopsies was detected in all but one patient with available gastric tissue. Using the same methodology, an *IgH* rearrangement of the same size was detected in the blood of 28 of 62 (45%) patients. Because the detection of a monoclonal product in the blood per se cannot definitely prove clonal identity between blood and stomach [35], we further compared the sequences of the two compartments. Sequencing was performed in five randomly picked stage I patients with available blood and gastric tissue. Clonal identity between blood and stomach was proven in four of five patients. Although sequencing analysis was not performed in all patients who were IgH^+ in the stomach and blood, our findings indicate that a significant proportion of patients with localized GML actually have circulating lymphomatous disease. There is only one other study, by Bertoni et al. [36], describing a similar finding. However, those investigators did not further compare circulating and gastric clones [36]. Other investigators have compared clones in MALT lymphomas between two or more different sites, such as the spleen and stomach, stomach and blood, multiple mucosal sites, and bone marrow, and found the same infiltrating clone [37–40]. However, these cases did not represent localized MALT lymphomas. To our knowledge, this is the first study to specifically address the above question [41, 42]. It is of interest that, in our series, all stage II_1 patients (three of three) had a lymphomatous clone in the blood, versus 42% of stage I GML patients, although the difference was not significant. Thus, stage II_1 GML cases tended to display occult blood disease more frequently than stage I cases. One could postulate lymphomatous spillover into the blood with growing tumor burden. In accordance with our findings, Thieblemont et al. [11] and Du et al. [11, 43] showed that early-stage GML is already disseminated at diagnosis when a more thorough staging investigation is applied or when biopsies from multiple sites of the intestinal tract are studied by molecular methods. The above studies

Table 2. Results of sequencing study

Patient no.	Blood				Stomach				Comparison between blood and gastric sequences
	VHGS	Homology to germline sequence	R/S	*p*-value[a]	VHGS	Homology to germline sequence	R/S	*p*-value[a]	
1	V2–5*10 D3–22*01 J4*02	100%	–	–	V2–5*10 D3–22*01 J4*02	100%	–	–	Same Igs
2	V3–74*01 D2–2*01 J4*02	96.1%	FR, 3/2 CDR, 5/1	FR, 0.013 CDR, 0.005	V3–74*01 D2–2*01 J4*02	94.4%	FR, 6/4 CDR, 5/1	FR, 0.028 CDR, 0.034	Same Igs Same Igs
3	V3–21*02 D5–24*01 J4*02	96.8%	FR, 2/2 CDR, 2/1	FR, 0.048 CDR, 0.158	V3–21*02 D5–24*01 J4*02	95.49%	FR, 2/2 CDR, 2/1	FR, 0.049 CDR, 0158	
4	V3–11*03 D3–3*01 J4*02	98.26%	–	–	V3–11*03 D3–3*01 J4*02	98.26%	–	–	Same Igs
5	V3–48*03 D3–22*01 J4*02	95.83%	FR, 5/1 CDR, 4/2	FR, 0.08 CDR 0.04	V3–48*03 D3–10*01 J4*02	92.71%	FR, 9/7 CDR, 5/0	–	Different Igs

Abbreviations: CDR, complementarity determining region; FR, framework region; R/S, replacement/silent mutation; VHGS, variable heavy gene segment.
[a]*p*-value for R/S mutation in FR and CDR according to multinomial distribution analysis.

Table 3. Treatment and response in Hp^+ patients who failed antibiotics and Hp^- patients

Treatment	Hp^+ patients ($n = 41$)		Hp^- patients ($n = 11$)	
	n	CR	*n*	CR
CT or CT + RT	30	16	5	2
CT + S	4	4	4	4
S	5	4	2	2
RT	1	1	–	–
Unknown	1	–	–	–

Abbreviations: CR, complete response; CT, chemotherapy (chlorambucil or cyclophosphamide, vincristine, and prednisone); Hp, *Helicobacter pylori*; R, rituximab; RT, radiotherapy; S, surgery.

have investigated the concomitant occurrence of MALT lymphoma in extranodal sites other than the stomach in GML patients. However, circulating occult blood disease was not addressed in any of those studies. Based on our findings, in which a significant proportion of our patients with localized GML had a circulating clone, one could postulate that the malignant cells can adhere to different extranodal sites and develop into a true lymphoma when the appropriate adhesion molecules and local addressins are encountered [44–48]. The significance of the circulating clone has not been elucidated.

Using molecular techniques, it has been demonstrated that, following anti-Hp therapy, there is a persistence of the gastric B-cell monoclonal population in about half of the complete responders [15]. We could hypothesize that the circulating clone might explain the failure of local treatment in a number of patients, or a subsequent relapse in another site after local therapy. However, this hypothesis cannot be supported by our data, because there was no difference in the CR rate with anti-Hp treatment between blood IgH^+ and IgH^- patients. Moreover, the presence of occult blood disease did not correlate with the relapse rate or pattern of relapse among patients who received local treatment. In addition, the presence of blood *IgH* rearrangement did not influence PFS or OS for the whole patient population. Thus, the clinical significance of the circulating clone is not apparent from this study. However, relapses in this indolent disease are rare events, and a higher number of patients is needed to make such correlations.

Another interesting finding was the more frequent usage of the *VH3* gene family by blood and gastric clones. A similar finding was recently reported by Sakuma et al. [49],

who analyzed *VH* gene usage in gastric biopsies of GML patients. They found that the most frequently used genes belonged to the *VH3* gene family and specifically *VH3–23* and *VH3–30*. These findings suggest that GML is derived from highly restricted B-cell subsets probably resulting from specific antigenic stimulation, such as with Hp [50–54]. In our study, patients with Hp infection were more frequently monoclonal in the blood than patients without Hp infection, but the difference was not statistically significant; therefore, occult blood lymphomatous disease cannot be attributed to Hp presence. Mutational analysis revealed that the gastric clones of three patients were mutated. In two of these, the sequences in the blood and stomach were identical. Statistical analysis using the multinomial distribution model revealed statistically significant *p*-values for the *CDR* in the identical blood and gastric clones of one patient and in the blood sequence of one patient with different clones, meaning that there is probable antigen selection pressure accounting for these mutations [53, 55]. Concerning the patient with different blood and gastric clones, blood PCR resulted in the detection of two different clonal products, and sequencing analysis rendered only one productive sequence different from the gastric clone. We can only hypothesize that the blood clone can be attributed to the oligoclonal expansion also evident in other lymphoma patients, although we were unable to detect a second productive sequence by our methodology [56].

In conclusion, we demonstrated that early-stage GML does not seem to be a truly localized disease, because almost half of our patients had a circulating clone probably identical to the clone in the stomach. However, the clinical significance of the blood clone is not yet clear. In the era of monoclonal antibodies, the early detection of occult blood disease and the appropriate intervention need to be further studied [57, 58].

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HIGH PREVALENCE OF DEMENTIA AND COGNITIVE IMPAIRMENT IN INDIGENOUS AUSTRALIANS

To the Editor: We read with interest article by Smith et al.,[1] who concluded that the prevalence of dementia and cognitive impairment is substantially higher among Indigenous than non-Indigenous Australians.

The background prevalence of *Helicobacter pylori* (Hp) urea breath test positivity in Indigenous Australians is 76%; the prevalence in the remote rural community is 91%, compared to 60% in the urban community.[2] Although degenerative diseases including dementia and cognitive impairment have an increasingly high impact in the aged population, their association with Hp infection (Hp-I) has only recently been addressed.[3,4]

A relationship between dementia and Hp-I appears to exist based on comparable data. To clarify, both diseases mainly affect old people in the developed world and Hp-I has been implicated in a variety of extra-digestive vascular conditions including functional vascular disorders, hypertension, atherosclerosis, and ischemic heart and cerebrovascular disorders. These also appear to be risk factors for dementia, mainly by impairing blood–brain barrier, a common denominator associated with dementia including Alzheimer disease (AD). These conditions contribute to the clinical manifestations and worsening of AD.[5]

In the nervous system, Hp-I may be associated with autoimmune sequelae development observed in peripheral neuropathies and AD.[5] We have documented a high prevalence of Hp-I in Greek patients with AD, establishing a significant relationship between Hp-I and AD confirmed by histology.[3] In a subsequent study, we found a high prevalence of Hp-I in Greek patients with mild cognitive impairment.[4] We also found an increased Hp-specific IgG antibody level in the CSF of patients with AD and this titer correlated with the degree of disease severity.

It would be interesting to know if Smith et al.[1] considered comparable data in their Indigenous Australian participants who would be expected to present with high Hp-I prevalence. Such data may be important considering the many ways that Hp-I could influence the pathophysiology of dementia.

Specifically, Hp-I promotes arteriosclerosis through induction of platelet-leukocyte aggregation, via autoimmune processes against endothelial cells, or by increased homocysteine blood levels due to decreased folic acid and cobalamin. It also produces reactive oxygen metabolites participating in neuronal oxidative damage loss. Further pathogenetic mechanisms include conversion of fibrinogen into fibrin, induction of molecular mimicry by the saccharide part of lipopolysaccharides of Hp with host antigens, release of proinflammatory and vasoactive agents, and induction of apoptotic processes.[5]

J. Kountouras, E. Gavalas, M. Boziki, C. Zavos, G. Deretzi, N. Grigoriadis, E. Tsiaousi, P. Katsinelos, D. Chatzopoulos, D. Tzilves, G. Kouklakis, Thessaloniki, Greece

Disclosure: The authors report no disclosures.

Reply from the Authors: We thank Dr. Kountouras and colleagues for their interest in our article.[1] We did not test for Hp-I during our study, yet we recognize that Hp-I is prevalent in Indigenous Australians.[2]

There are a number of plausible explanations for an association between Hp-I and cognitive impairment but not all imply causality. Dr. Kountouras et al. consider possible mechanisms by which Hp-I may be a contributing factor to dementia and cognitive impairment.

Based on current knowledge, such a relationship cannot be excluded. One theory is that the effect of atrophic gastritis (caused by the infection) on levels of vitamin B12, folate—and thus homocysteine—may increase the risk of cognitive impairment through vascular disorders.[3] Others have demonstrated that folate and B12 nutritional deficiency and elevated homocysteine levels in Indigenous Australians[6] and diminished levels of vitamin B12 and elevated homocysteine have been associated with cognitive impairment.[7]

Although it was not one of our research objectives, the association between the high prevalence of Hp-I and dementia in Indigenous Australians is intriguing and requires further investigation.

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Disclosure: The authors report no disclosures.

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CEREBELLAR LEUKOENCEPHALOPATHY: MOST LIKELY HISTIOCYTOSIS-RELATED

To the Editor: I read the article by van der Knaap et al.[1] with interest.

After 6 months of progressive dysarthria, dysphagia, and ataxia, a 59-year-old Caucasian man was transferred to our facility for further workup for progressive leukoencephalopathy. He had stage III melanoma and a left axillary dissection 5 years previously. CT chest and fluorodeoxyglucose PET done 2 months after onset of neurologic symptoms showed increased uptake in the left axilla. Pathology from a repeat axillary dissection was negative for recurrence of melanoma.

A CD68 positive, histiocytic infiltrate was seen in the lymphoid tissues, but was believed to be a reactive process related to prior surgery (CD1a, S100 testing not performed). Surgical pathology from stereotactic biopsy of the right cerebellar white matter performed 5 months after onset of neurologic symptoms revealed demyelination and scattered perivascular and parenchymal CD8-positive lymphocytes. There was no evidence of neoplasia, infection, vasculitis, or granulomatous disease.

Laboratory evaluations for paraneoplastic, inflammatory, infectious, nutritional, metabolic, and degenerative disorders were unremarkable. A lumbar puncture including cytology revealed mildly elevated protein. The patient had refractory hyponatremia and hyperprolactinemia suggesting hypothalamic-pituitary dysfunction. Brain MRI revealed T2 hyperintensities in the pontine tegmentum, middle and superior cerebellar peduncles, and in the cerebellar white matter, predominantly in the dentate hilus with no significant cerebellar atrophy. These areas enhanced heterogenously. Cortical T1 hyperintensities were seen in the right mesial and anterior temporal region with no mass effect.

The patient received pulsed IV steroids, 6 weeks of oral steroids, and 2 g per kilogram of IV immunoglobulin. The patient continued to worsen and became bedbound. Two months after admission, MRI revealed worsening of the patient's leukoencephalopathy with extension to the midbrain, basis pons, and upper medulla. Repeat fluorodeoxyglucose PET revealed diffuse uptake in the right temporal lobe, brainstem, cerebellum, spleen, adrenals, and kidneys consistent with a systemic inflammatory process. The patient deferred any further evaluation and died 1 year after the onset of neurologic symptoms.

The neuroimaging and neuropathology in our case, particularly the involvement of the dentate hilus and the supratentorial T1 hyperintensities, are similar to those patients observed in the study by van der Knaap et al. The histiocytic infiltrate seen on the axillary biopsy was considered benign but a histiocytosis was not excluded.

I believe our patient had a paraneoplastic leukoencephalopathy, most likely histiocytosis-related, followed by systemic involvement with a more fulminant clinical course than seen by the authors.[1]

Brett J. Theeler, Tacoma, WA

Disclosure: The author reports no disclosures.

Reply from the Authors: We appreciate Dr. Theeler's interest in our article. We agree with the conclusion that his patient had a clinical picture and MRI findings similar to our patients.[1]

In his patient, axillary dissection revealed a histiocytic infiltrate in the lymphoid tissues; biopsy of the right cerebellar white matter demonstrated myelin loss and scattered perivascular and parenchymal CD8-positive lymphocytes, without evidence of neoplasia, infection, vasculitis, or granulomatous disease. These histopathologic results are consistent with those seen in our patients.[1] The findings in Dr. Theeler's patient further confirm our conclusion that cerebellar leukoencephalopathy can be histiocytosis-related.[1]

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Primary Open-Angle Glaucoma

TO THE EDITOR: With regard to the review article on primary open-angle glaucoma by Kwon et al. (March 12 issue),[1] we believe that some discussion of the possible role of vascular factors in the pathophysiology of glaucomatous optic neuropathy is necessary.

Investigators in the Barbados Eye Studies reported that baseline vascular risk factors, including decreased systolic blood pressure and decreased systolic, diastolic, and mean ocular perfusion pressure, can influence the risk of open-angle glaucoma. Specifically, low ocular perfusion pressure doubled the risk of glaucoma in that population.[2] Investigators in the Early Manifest Glaucoma Trial reported that baseline predictors of progression of open-angle glaucoma include decreased ocular systolic perfusion pressure, a history of cardiovascular disease, and decreased systolic blood pressure.[3] Another study showed that a diastolic blood pressure of less than 90 mm Hg due to antihypertensive treatment is associated with increased optic-nerve cupping and a decreased rim area of the optic disk in subjects without glaucoma.[4]

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TO THE EDITOR: Regarding the review article by Kwon et al.: it appears that *Helicobacter pylori* infection is associated with various risk factors for primary open-angle glaucoma.[1,2] Moreover, an association between *H. pylori* infection and primary open-angle glaucoma has been found in a Greek cohort, and levels of *H. pylori* infection–specific IgG antibodies are increased in the aqueous humor in patients with primary open-angle glaucoma; the concentration of this antibody correlates with the degree of vertical cupping, possibly indicating the severity of glaucomatous damage.[3] Similar observations[4,5] have been made in China, India, Turkey, and Iran.

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THE AUTHORS REPLY: We agree with Grzybowski and Harris that systemic and local vascular factors play an important role in the pathogenesis of primary open-angle glaucoma, as shown by several large epidemiologic studies, both cross-

sectional and longitudinal. In a study involving clinic populations, an author of our review article and others reported that patients with normal-tension glaucoma and patients with anterior ischemic optic neuropathy who showed progressive visual-field deterioration were more likely to have nocturnal, systemic hypotension than patients with stable visual fields.[1] Because of space constraints, we confined the discussion in our review article to the mechanisms of elevated intraocular pressure and resultant changes to the cells and structures of the optic-nerve head (or optic disk).

In reply to the comments by Kountouras et al.: the association between *H. pylori* infection and glaucoma remains controversial. Since the initial reports of the association observed in a Greek cohort involving 41 study subjects and 30 control subjects, a larger independent study in Canada (involving 97 study subjects and 94 control subjects)[2] and another study in Israel (involving 51 study subjects and 36 control subjects)[3] showed that *H. pylori* seropositivity in the study cohort was not significantly greater than that of controls. A study from South India involving 50 study subjects and 50 control subjects showed that the level of anti–*H. pylori* IgG antibodies in the serum, but not in the aqueous humor, was significantly elevated in patients with primary open-angle glaucoma as compared with that of controls.[4] The negative finding in the aqueous humor is in contrast to the significantly elevated level of IgG detected in the aqueous humor in patients with primary open-angle glaucoma in an earlier study from Greece involving 26 study subjects and 31 control subjects.[5] We acknowledge that the hypothesis concerning the contribution of *H. pylori* infection to the pathogenesis of glaucoma through a cellular or humoral immune response remains an intriguing possibility. However, to date, independent studies involving larger cohorts have not corroborated the conclusions of the initial reports.

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Preservation of Fertility in Patients with Cancer

TO THE EDITOR: With regard to the review article by Jeruss and Woodruff (Feb. 26 issue)[1]: we appreciate the increased attention to options for the preservation of fertility in people with cancer; however, it is important to inform readers of the evidence-based guidelines on this topic that were published in 2006 by a committee convened by the American Society of Clinical Oncology (ASCO).[2] The ASCO guidelines were based on a systematic review of the literature and formal procedures for guideline creation, including composition of the committee, critique by outside experts, and review by ASCO administrative bodies. Per ASCO policy, these guidelines will also be updated periodically with the use of the same rigorous procedures. Many of the specific recommendations in the review by Jeruss and Woodruff are quite controversial, and their approach should be recognized as one among many in an evolving field.

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TO THE EDITOR: In their review, Jeruss and Woodruff state that "children undergoing chemother-

Letter to the Editor

Asian J Ophthalmol 2009; 11:46-47

Association of *Helicobacter pylori* Infection and Open Angle Glaucoma: Comparison of Greek and Chinese Data

Dear Editor,

Hong et al should be commended for their effort to investigate the association between *Helicobacter pylori* infection and the occurrence of open-angle glaucoma in China by using the ^{13}C-urea breath test.[1] The ^{13}C-urea breath test is the non-invasive diagnostic test of choice, having a significantly higher diagnostic accuracy than serology, particularly for older subjects.[2] However, Hong et al[1] claim that, unlike our own results,[3] they did not find an association between *H pylori* infection and the stage of glaucoma as estimated by mean visual defect and cup-disc ratio in 13 and 11 patients who were *H pylori*–positive and –negative, respectively.

In our study,[3] mentioned by Hong et al,[1] we found that only mean vertical cupping correlated significantly with the titre of anti-*H pylori*–specific immunoglobulin G in the aqueous humour, thereby reflecting the severity of glaucomatous damage. Therefore, it would be of interest to know whether the authors performed similar measurements in the aqueous humour and not in serum to obtain comparable data to ours and support their argument on the stage of glaucoma. Moreover, it should be noted that measurements in such a small number of patients means that their study is underpowered and of limited value, and thus further recruitment should be encouraged. It would be of particular interest if the authors could report any differences or similarities with our own data with respect to mean intraocular pressure, short-term fluctuation, or corrected loss of variance between *H pylori*–positive and –negative patients.

From another interesting viewpoint, because *H pylori* prevalence is significantly lower in patients with gastro-oesophageal reflux disease (GORD) from East Asia, including China, than in those from western countries, possibly indicating a protective role against GORD,[4,5] comparable data did not explain its association with glaucoma development in the Chinese population.

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Comment

Dear Editor,

Kountouras et al have raised a number of issues regarding our article.[1]

They found the titre of anti–*Helicobacter pylori* antibody in the aqueous humour might reflect the severity of glaucomatous damage in patients with primary open angle glaucoma (POAG) by aspirating aqueous humour at the beginning of glaucoma surgery.[2] In our series, the intraocular pressures (IOPs) of 28 eyes were well controlled by medications without surgery.[1] We therefore had difficulty getting aqueous humour samples from these patients.

Serologic testing by enzyme-linked immunosorbent assay is a method for detecting current and old *H pylori* infection, while ^{13}C-urea breath test is a good screening method for current *H pylori* infection. Therefore, we recorded the IOP, visual field investigation, and ^{13}C-urea breath test during 1 week. We supposed that mean deviation may be an important parameter to differentiate between

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H pylori–positive and –negative patients, since different patients have different target IOPs.

The small number of patients may limit the significance of our findings. However, this is partly because the incidence of PEAG is much lower than angle closure glaucoma in Asia.[3,4]

The infection rate of *H pylori* was strongly associated with age and socioeconomic conditions. *H pylori* infection is endemic in China.[5] The infection rate in our study (20.8%)[1] is similar to those in cities in which the socioeconomic conditions are almost same as in Beijing.[5]

Kountouras et al claimed that there may a protective role against gastro-oesophageal reflux disease (GORD) in China and this protective role may be associated with glaucoma development in the Chinese population. This was not a part of our study. We may do further research into this, but we do not think that the lower *H pylori* prevalence in gastroesophageal reflux disease (GERD) in China has a relationship with the prevalence of *H pylori* in the general population.

Some authors have demonstrated that glaucoma might be related to *H pylori* infection,[2] while others think that there is no relationship between glaucoma and *H pylori* infection.[7] Further research is needed.

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Letter to the Editor

A concept of *Helicobacter pylori* and stress-secreted mast cells' potential involvement in brain metastases

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We read with interest the paper by Theoharides et al. (2008), who concluded that mast cells can be stimulated by corticotropin-releasing hormone, secreted under stress, to release mediators including histamine, interleukin (IL)-8, tryptase and vascular endothelial growth factor (VEGF), which disrupt the blood–brain barrier (BBB) permitting metastases of lung and mammary adenocarcinomas.

Helicobacter pylori infection (*Hp*-I) appears to be a common denominator of lung, mammary and gastrointestinal (GI) tract cancers contributing to the mentioned stress–mast cell–BBB disruption–brain metastases sequence of these malignancies.

Indeed, apart from GI malignancies (mainly gastric cancer), *Hp*-I has been involved in lung and breast cancers (Prelipcean et al., 2007; Moss and Malfertheiner, 2007; Kountouras et al., 2004), mainly via induction of the oncogenic growth factor gastrin which contributes to lung and breast carcinogenesis and metastasis (Yonemori et al., 2005; Gugger and Reubi, 1999); an elevated pro-gastrin-releasing peptide level before prophylactic cranial irradiation appears to be significantly related to the first failure event due to brain metastases and survival of lung carcinoma (Yonemori et al., 2005).

Physical and psychological stresses are accepted as triggers and/or modifiers of various GI disorders clinical course. Stress can also synergize with other pathogenic factors such as *Hp* to produce upper GI malignancies. The brain–gut axis provides the anatomical basis through which emotions and environmental influences modulate the GI function through the regulation of GI immune system and mucosal inflammation; in this regard, mucosal mast cells – at cellular level – and corticotropin releasing factor (CRF), at molecular level, appear to play a critical role (Caso et al., 2008).

Specifically, via oxidative stress, *Hp*-I causes inflammation, accumulation of reactive oxygen species (ROSs) and oxidative DNA damage in the gastric mucosa; ROSs play an important role in *Hp*-related gastric carcinogenesis (Kountouras et al., 2001). In addition, a series of factors have been implicated in inducing BBB disruption, including inflammatory mediators (e.g., cytokines and chemokines induced by *Hp*-I), and oxidative stress (Kountouras et al., 2008; Keep et al., 2008). *Hp* could indirectly affect the brain and other target organs such as the heart, through the release of numerous cytokines such as tumor necrosis factor (TNF)-α acting at distance; TNF-α is involved in BBB disruption through a mechanism involving matrix metalloproteinase upregulation (Candelario-Jalil et al., 2007), thereby possibly contributing to brain metastases. As in the case of BBB disruption, extracts of *Hp* (*Hp*-E)-induced microvascular dysfunction appears to be a consequence of interstitial and intravascular cell–cell interactions. *Hp*-E increase leukocyte adherence and emigration, and microvascular albumin leakage. *Hp*-E also elicit perivenular mast cell degranulation and the formation of platelet–leukocyte aggregates within post-capillary venules (Kurose et al., 1994). In this respect, *Hp*-induced IL-8 (acting as an angiogenic factor) and VEGF (contributing to *Hp*-related gastric carcinogenesis) may be independent and important prognostic factors in human gastric carcinomas (Kountouras et al., 2008).

Noticeably, as in the case of *Hp*-I, oxidative stress activates the gene expression of a specific cytokine pattern in mast cells through an apurinic/apyrimidinic endonuclease-1/redox factor-1-dependent pathway (Frossi et al., 2003). *Hp*-induced cytotoxin VacA (Kountouras et al., 2001) exhibits chemotactic activities to the bone marrow-derived mast cells (BMDMCs) and induces BMDMCs to produce proinflammatory cytokines (Supajatura et al., 2002). Densities of mast cells, proliferating cell nuclear antigen-positive mast cells and mast cell growth factor (stem cell factor)-positive cells are significantly greater in *Hp*-positive than negative subjects (Bamba et al., 2002). In addition, the *Hp*-induced neutrophil-activating protein recruits

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leukocytes from the vascular lumen and activates neutrophils, monocytes, and mast cells. Moreover, *Hp*-induced chemokine IL-8 have stimulatory effects on the histamine release from gastric mucosal mast cells; IL-8 and *Hp* soluble factors may accelerate inflammation of the gastric mucosa through histamine release from mast cells (Yakabi et al., 2002). Therefore, mast cells are actively involved in the pathogenesis of *Hp*-associated gastric pathologies; the number of mast cell-associated epithelial cells correlates with a high number of apoptotic epithelial cells (Hofman et al., 2007). Mast cell chymase may be a significant mediator in the inflammatory processes of human *Hp*-induced gastritis (Matsuo et al., 2003). Moreover, tryptase is linked to *Hp*-I, and plays a part in *Hp*-induced gastric pathologies; tryptase might reflect an indirect link between *Hp*-I, gastrin release, and the function of mast cells (Plebani et al., 1995).

Chronic infection of C57BL/6 mice and humans with *Hp* might induce repopulation of the stomach with bone marrow-derived stem cells (BMDSCs) and possibly BMDMCs that may facilitate gastric cancer progression (Kountouras et al., 2008).

Hp also induces release of other cytokines (IL-1, IL-6), eicosanoids (Gavalas et al., 2007), endothelin-1 (Kountouras et al., 2007), or possibly CRF (distributed widely in the stomach) (Wakabayashi et al., 1985) involved in the mentioned mast cell pathophysiology of malignancies (Theoharides et al., 2008; Theoharides, 2008).

The observed *Hp* and/or gastrin-induced activation of GI tract and peritoneal cavity masts cells, and primary cultured mast cells, generated from CD34(+) progenitors in the presence of stem cell factor and IL-6 (Mahjoub et al., 2007; Montemurro et al., 2002; Kulka et al., 2008), may also induce BBB disruption through the release of several multifunctional cytokines (Esposito et al., 2002; Theoharides et al., 2007), including TNF-α acting at distance (BBB) and leading to potential *Hp*-related brain metastatic disease.

Summarizing, *Hp*-I might be involved in the mentioned stress–mast cell–BBB disruption–brain metastasis sequence of GI and *Hp*-related lung and breast malignancies. It would thus be interesting to know if Theoharides et al. (2008) have considered comparable data in their studies. Such data appear to be important, because, *Hp* simple eradication regimens might also prevent brain metastases of *Hp*-related malignancies.

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Anti-*Helicobacter pylori* antibody responses specific for VacA do not trigger primary biliary cirrhosis-specific antimitochondrial antibodies

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Recently, Goo *et al.* [1] reported the induction of primary biliary cirrhosis (PBC)-like pathology in a C57BL/6 mouse infected with *Helicobacter pylori* and speculated that anti-VacA antibodies can induce experimental disease and antibodies against the dominant pyruvate dehydrogenase complex E2 subunit (PDC-E2) autoantigen, through a mechanism of molecular mimicry.

We have investigated in detail the pathogenic role of molecular mimicry involving microbial agents such as *H. pylori* and PDC-E2 autoepitopes [2–5] and we would like to raise a few points:

We have noted that Goo *et al.* [1] have not tested whether anti-PDC-E2 antibodies are present in the mouse with PBC-like pathology.

We have tested 70 patients with PBC (50 anti-PDC-E2 positive and 20 anti-PDC-E2 negative) [3,4] and 100 demographically matched controls (70 with chronic hepatitis C and 30 normal), all from Greece, for reactivity to VacA of *H. pylori* by immunoblotting (Euroimmun, Lübeck, Germany). The presence and levels of IgG class anti-VacA antibodies did not differ between anti-PDC-E2 positive and PDC-E2 negative PBC cases (17 of 50, 34% vs. 6 of 20, 30%) or between PBC patients and controls (33 vs. 31%).

Solid-phase inhibition experiments in anti-VacA/PDC-E2 double reactive cases while abolishing reactivity to PDC-E2, left unaffected reactivity to VacA *H. pylori*. The reciprocal experiment using VacA as inhibitor left unchanged anti-PDC-E2 antibody reactivity.

Through a BLAST2p protein–protein database search, we have found insignificant similarities between VacA of *H. pylori* and human PDC-E2 (30–61% homology for the best five matches). None of the VacA/PDC-E2 mimics involved the – critical for antibody binding – PDC-$E2_{212–226}$ core epitopic region.

Our data suggest that anti-VacA antibody responses do not cross-react and are not associated with PBC-specific anti-PDC-E2 responses. Anti-VacA *H. pylori* antibodies are most likely irrelevant to the pathogenesis of PBC.

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Signature genes for both hepatoblastoma and hepatocellular carcinoma

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A major goal in current hepatocellular carcinoma (HCC) research is to define molecular or gene signatures that govern initiation, maintenance and progression of the malignant tumours. The gene signature should be helpful in classifying tumour stages and predicting prognostic outcomes, such as metastasis, patient survival rate and recurrence of the tumours after resection. It is also highly desirable to use the gene signature to design targeted therapies or the so-called personalized medicine.

Most recently, Cairo and colleagues [1] reported signature genes for hepatoblastoma (HB), an infant liver tumour that is clinicopathologically distinctive