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Relationship between *Helicobacter pylori* seropositivity and *Hyperemesis gravidarum* with the use of questionnaire

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Aim. **The aim of the present study was to investigate the relationship between *Helicobacter pylori* (HP) infection and *Hyperemesis gravidarum* (HG) by using a questionnaire.**

Methods. **Twenty-five pregnant women with *HG* and 85 asymptomatic pregnant women (aged 14-40) of matching gestational age were enrolled between October 2004 and January 2006. Anti-HP immunoglobin G (IgG) serum antibody was tested to establish seropositivity. In our study we used a multi variable questionnaire (name, age, gravida, number of vomits daily etc.). The results were analyzed using χ^2 and Mann-Whitney U- test.**

Results. **The prevalence of HP infection was 56% (14 of 25) among patients with *Hyperemesis gravidarum* and 48.2% (41 of 85) among control subjects (P>0.05, χ^2 test). In the same study the HP seropositivity is not related to age of the woman (50%, 55 of 110 were HP positive, P>0.05 χ^2 test) but there was a significantly association between number of deliveries and HP seropositivity (primigravida [+] 34.2% *versus* 65.8%, multigravida [+] 6.1% *versus* 38.9%, P<0,05 χ^2 test). The history for gastrointestinal problems of the tested women and their husbands is not related to HP seropositivity(38.1% positive for HP and with gastrointestinal disorders she/he or both *versus* 61.8% positive and without problems both, P>0.05, Mann-Whitney U- test). Finally there was no relativity between the number of vomits daily and the HP seropositivity (48.2% with HP [+] and 0-3 vomits daily *versus* 56% with HP [+] and 4-8 vomits daily, P>0.05 χ^2).**

Conclusions. **Our findings do not support any direct correlation between HP seropositivity and *Hyperemesis gravidarum*, number of vomits daily, age of woman, history for gastrointestinal problems but only with the number of deliveries.**

Key words: **Helicobacter pylori - Hyperemesis gravidarum - Pregnancy.**

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The first trimester of pregnancy is usually characterized by disorders of the gastrointestinal system, such as nausea and morning sickness. This is observed in a percentage of 50-90% of pregnant women. The abovementioned symptoms usually cease spontaneously around the 14th-16th week of pregnancy.[1] *Hyperemesis gravidarum* (HG) is a complication observed in a smaller number of women (0.3-2%), featured by the presence

TABLE I.—*Questionnaire for the relationship of HP and HG.*

Name-Surname	Age	Gravida (way of delivery)	Presence of gastrointestinal disorders of the woman and the partner	Number of daily vomits in previous pregnancies	Number of daily vomits during the present pregnancy 0-1 2-3 4-5 6-8 >8	Quantitative of IgG HP

of several vomits daily resulting progressively in morbid situations such as weight loss, hypokaliemia, dehydration, acidosis from starvation, alkalosis from loss of hydrochloric acid in vomitus, while in some cases jaundice may appear.

Its origin is unknown, it appears more frequently in primigravida women and is probably related to high estrogen, progesterone and free human chorionic gonadotropin (hCG) values. Psychological reasons can also lead to the above mentioned condition.[2] During the last 10 years studies have been carried out to observe the correlation between HG and the modification sustained by the gastric pH during pregnancy.

The aim of the present study was to investigate and document a presumptive association between *Helicobacter pylori* (HP) seropositivity and hyperemesis, and more specifically with the number of vomits daily, all documented by the use of a questionnaire.

Materials and methods

The material consists of a random sample of 110 women, between 14 and 40 years, in the general hospital of Kalamata from October 2004 to January 2006, 25 of these women presented symptoms and clinical aspect of HG (study group I). Inclusion criteria were: number of vomits above 3 daily; weight loss about 3 kg or more; presence of chetonic corps in the urines, and gestational age 8-14 weeks. Cases in which the cause of the vomits were other diseases such as hyperthyroidism or psychological disorders were excluded from the study. The control group II included the remaining 85 asymptomatic pregnant women. In order to track down HP during pregnancy several diagnostic tests have been used in the past as the PCR/IgG ratio,[3] the use of Urea Breath's Test,[4] and gastroscopy.[5]

In the present study, for the localization of HP specific serum antibodies immunoglobulin G (IgG) directed against HP by a commercial anti-IgG enzyme linked immunosorbent assays (ELISA) kit (Serion, Germany) were used. A value ?45 U/mL was considered positive result, whereas a value of ≤44 U/mL was considered a negative result All speciments were collected at the same time of gestational age both at the control group and at the study group (12th-14th week of gestation). They were all centrifuged and placed in a -70° C temperature. For first time, a questionnaire was utilized in parallel; such questionnaire included the following data: name, age, parity (and way of delivery: labour or cesarean section), family history concerning gastrointestinal disorders of the woman (before that pregnancy or in previous pregnancies) and of the partner, number of daily vomits in previous pregnancies if present, number of daily vomits during the present pregnancy, quantitative assessment of immunoglobulins IgG HP. The patient's land of origin was also registered. In terms of the number of vomits at the present pregnancy a dissociation was made among the following: 0-1/2-3/4-5/6-8/>8 (Table I). The aim of this dissociation was to observe whether there is or not a relationship between HP seropositivity and the number of vomits that a woman has daily.

All women were examined at approximately 8 weeks of gestation with documented embryonic heartbeat, and then invited again at a gestational age between 12 and 14 weeks for the blood test and to fill in the questionnaire. Women with a first trimester miscarriage were excluded from the study. Twenty-two out of the 25 women of group I

TABLE II.—*Obstetric charateristics of study and control group.*

	Control group(II) N.=85 (77.2%)	Study group(I) N.=25 (22.7%)
Age (range 14-40)	27	27
Primigravida (N.)	28 (32.9%)	11 (44%)
Multigravida (N.)	57 (67.1%)	14 (56%)
Gestation week (8-14w)	11 w	11 w
IgG HP (+)	41 (48.2%)	14 (56%)
IgG HP (-)	44 (51.8 %)	11(44%)

were attended in our hospital. The obstetric characteristics of both study and control groups are presented in Table II. The results were tested for statistical significance by a χ^2 test and Mann-Whitney U- test and they were evaluated with 95% confidence interval.

Results

There is no consistent difference between the control group II (48.2%, 41 pregnant women out of 85) and the tested group I (56%, 14 pregnant women out of 25) for the HP seropositivity (P=0.494 χ^2). In parallel, no association between the number of vomits daily and HP seropositivity was observed (P=0.868 χ^2, 48.2% with 0-1 vomits daily and HP [+] *versus* 51.4% with the same number of vomits and HP [-], 47% with 2-3 vomits daily and HP [+] *versus* 52.9% with the same number of vomits and HP [-], 42.8% with 4-5 vomits daily and HP [+] *versus* 57.1% with the same number of vomits and HP [-], 66.6% with 6-8 vomits daily and HP [+] *versus* 33.3% with the same number of vomits and HP [-], 58.3% with up to 8 vomits daily and HP [+] *versus* 41.6% in the same situation and HP [-]). No statistical significance is proven by the fact that the history for gastrointestinal problems of the tested women and their husbands is not related to HP seropositivity during pregnancy(P=0.561 Mann-Witney U-test). From 5 women with gastrointestinal disorders both she and her husband only 4 (80%) were HP (+), from 21 women who only them had gastrointestinal problems only 10 (47.6%) were HP (+), from 13 women that the husband had gastrointestinal disorders only 7 (53.8%) were HP (+) and finally from 71 women without gastrointestinal problems both she and her husband only 34 (47.8%) were HP (+).

Because of the wide of variation of age among the women tested, for the present study we also examined the possible association with a particular age range; such examination proved that HP positivity is not related to the age of the woman (P=0.071 χ^2). (33% 14-16 years old and H. pylori [+] *versus* 67% in the same age and HP [-], 0% 17-19 years old and HP [+] *versus* 100% HP [-], 58.3% 20-22 years old and HP [+] *versus* 41.7% HP [-], 42.3% 23-25 years old and HP [+] *versus* 57.7% HP [-], 52.6% 26-28 years old and HP [+] *versus* 47.4% HP [-], 73.3% 29-31 years old and HP [+] *versus* 26.7% HP [-], 37.5% 32-34 years old and HP [+] *versus* 62.5% HP [-], 57.1% 35-37 years old and HP [+] *versus* 42.9% HP [-] and finally 83.3% 38-40 years old and HP [+] *versus* 16.7% in the same age and HP [-]).

The only element that appears to be statistically significant is the number of deliveries and the presence of HP. In particular women who have given birth once showed less percentage of HP seropositivity compared to women who have given birth to more than two (P=0.00058 χ^2). (34.2% with 1 delivery and HP [+] *versus* 65.8% in the same condition and HP [-], 63.4% with 2 deliveries and HP [+] *versus* 36.6% HP [-], 58.3% with 3 deliveries and HP [+] *versus* 41.7% HP [-], 100% with 4 deliveries and HP [+] *versus* 0%, 50% with 5 deliveries and HP [+] *versus* 50% HP [-]and in the end 0% with 6 deliveries and HP [+] *versus* 100% in the same situation and HP [-]).

Discussion

HP is a non invasive, non-spore forming s-shaped gram-negative rod measuring approximately 3-5 x 0.5 μm. HP is very importantas it is present in 70% to 90% of patients with duodenal ulcers and in about 70% of those with gastric ulcers.[6] During the last few years the presence of HP has been considered responsible also for gastric cancer.[7] The most important observation of the present study is

that there is no association between the symptoms of hyperemesis and the HP IgG concentration in the serum. Such result can be observed in several other studies, which unrelated to the type of HP identification, had the aim to determine the cause-effect relation of the microbe and the HG, even if results are different. Recent studies claim that there is no interference between HP infection and gastrointestinal disturbances during pregnancy, [8, 9] whereas other studies ascribe to it an important association.[10, 11] Our effort focused on the registration, by the use of questionnaire, of the number of daily vomits during the first trimester of pregnancy and the possible association that it might have with HP. The results show that the number of vomits is not related with the presence of HP. The cause of HG remains unknown, but we can not rule out a possible role of HP in creating this situation.[12] In a recent study Shirin *et al.*[13] concluded that pregnant women who were HP positive were older and had more prior pregnancies and deliveries. The study of Blecker *et al.*[14] ended to the same conclusions, namely that HP positivity is related to the age of the patient.

In our study we elicited that HP seropositivity is not correlated to the age of the woman (in an age range between 14-40 years), whereas women who were pregnant for the first time were less frequently positive to HP (34.2% 13 out of 38) in regard to multiparous women (61.1% 44 out of 72). Simultaneously the history of the patients was collected through the questionnaire with reference to gastrointestinal disorders of both pregnant women and their husbands. It is well known that HP has a worldwide distribution and although the mode of acquisition and transmission is not clear, it appears to be acquired by the fecal-oral or oral-oral route. Transmission of HP among family members has also been discussed in the past.[15] Nevertheless, such a conclusion did not emerge from our study, so that family history could be associated to HP presence during pregnancy. The big question which still remains is how much the presence of HP is related to HG, and, if it is not the primary cause, how much it participates in further aggravating the gastrointestinal disturbances in the first trimester of pregnancy.

It is possible that some of the women had gastrointestinal problems before pregnancy, such as gastritis, esofagitis, regurgitum, and these were aggravated also because of the modifications in gastric pH. What remains is the prevention and mainly the eradication of HP after the end of pregnancy and lactation, in order to avoid similar problems during a subsequent pregnancy and because of the problems it usually causes. During the last few years studies as the one by Kitagawa *et al.*[16] refer to a possible horizontal transmission of HP from lactating mothers to their newborn children.

As for the treatment of the symptoms of HG with antiemetics, it remains under discussion, especially with reference to the first trimester of pregnancy. This is due to its still unproven safety for the embryo.[3] In the present study the treatment of all hospitalized patients (22 out of 25) included i.v. hydration, electrolyte adjustment and use of dimenhydrinate suppositories 1 or 2 daily. Multivitamin complexes were also used supportively. After a mean time of 72 hours women gradually reacquired the ability to eat small quantities of food. In 3 out of the 22 women which were hospitalized 1 intravenus dose of promethazine daily for a maximum period of 2 days was infused.

Conclusions

In conclusion, despite the fact that a high percentage (56%) of the subjects with HG were HP positive, the symptoms could not be directly correlated to the microbe because it was also found in a high percentage (48.2%) of women with no such symptoms during the first trimester of pregnancy. Neither the recording of daily vomit episodes was associated to the presence of HP. Consequently the role of HP in provoking or aggravating HG still remains under investigation. Anyway further efforts are needed (also ameliorating similar questionnaires with higher sensitivity) to establish the relationship between the HG pathological entity and the presence of HP and its treatment.

Riassunto

Correlazione tra sieropositività per Helicobacter pylori *e* Hyperemesis gravidarum *con l'utilizzo di un questionario*

Obiettivo. L'obiettivo di questo studio è stato quello di valutare la correlazione tra l'infezione da *Helicobacter pylori* (HP) e l'*Hyperemesis gravidarum* (HG) utilizzando un questionario.

Metodi. Tra l'ottobre 2003 e il gennaio 2006 sono state arruolate 25 donne gravide con HG e 85 donne gravide asintomatiche con età simile (14-40 anni). Per stabilire la sieropositività sono state testate le immunoglobuline G (IgG) sieriche anti-HP. Nel presente studio è stato utilizzato un questionario con variabili multiple (nome, età, parità, numero di episodi di vomito giornalieri, ecc.). I risultati sono stati analizzati utilizzando il test del χ^2 e il test-U Mann Whitney.

Risultati. La prevalenza dell'infezione da HP è stata del 56% (14 su 25) tra le pazienti con HG e del 48,2% (41 su 85) tra i soggetti di controllo (P>0,05, χ^2 test). Nello stesso studio la sieropositività per HP non ha correlato con l'età delle donne (50%, 55 su 110 erano HP positive, P>0,05 χ^2 test) ma vi è stata un'associazione significativa tra il numero di parti e la sieropositività HP [primigravida (+) 34,2% *versus* 65,8%, multigravida (+) 6,1% *versus* 38,9%, P<0,05 χ^2 test]. La presenza di problemi gastrointestinali nelle donne testate e nei loro mariti non ha correlato con la sieropositività per HP (38,1% di positività per HP e disturbi gastrointestinali nella donna, nel marito o in entrambi *versus* 61,4% di positività senza problemi in entrambi, P>0,05, test-U Mann Whitney). Infine non è emersa alcuna correlazione tra il numero di episodi di vomito giornalieri e la sieropositività per HP (48,2% con HP [+] e 0-3 episodi di vomito giornalieri *versus* 56% con HP [+] e 4-8 episodi di vomito giornalieri, P>0,05 χ^2).

Conclusioni. I dati non hanno evidenziato alcuna correlazione tra sieropositività HP e HG, numero di episodi di vomito giornalieri, presenza di problemi gastrointestinali, ma solo con la parità.

Parole chiave: Helicobacter pylori - Hyperemesis gravidarum – Gravidanza.

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Alzheimer's disease and *Helicobacter pylori* infection: Defective immune regulation and apoptosis as proposed common links

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Summary Although degenerative diseases of the central nervous system, including Alzheimer's disease (AD), have an increasingly high impact on aged population their association with *Helicobacter pylori* (*H. pylori*) infection has not as yet been thoroughly researched. Current *H. pylori* infection appears to induce irregular 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 the apoptotic neural tissue damage observed in neurodegenerative diseases including AD. An association between AD and *H. pylori* infection has been recently addressed by two studies. A higher seropositivity for anti-*H. pylori* immunoglobulin G antibodies in 30 patients with AD than in 30 age-matched controls was reported in one study; this serological test, however, has limitations because it does not discriminate between current and old infections. In the other study, by introducing the histological method (the actual gold standard) for diagnosis of *H. pylori* infection, we reported a higher prevalence of *H. pylori* infection in 50 AD patients than in 30 anemic controls. This pathogen may influence the pathophysiology of AD by promoting platelet and platelet–leukocyte aggregation; releasing various pro-inflammatory and vasoactive substances; developing cross-mimicry with host antigens; producing reactive oxygen metabolites and circulating lipid peroxides; influencing the apoptotic process; and increasing, through induction of atrophic gastritis, homocysteine, which contributes to vascular disorders implicated in endothelial damage and neurodegeneration.

Alzheimer's disease

Alzheimer's disease (AD) is a progressive age-related neurodegenerative disorder that is the most common form of dementia affecting ~20 million people worldwide (6–8% of the population

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aged 65 years and 30% of the population aged 85 years); the number of AD cases is expected to increase with increased life expectancy [1–5]. In its earlier stages, the disease is characterized by progressive impairment in memory, visuospatial skills, complex cognition, language, and personality. Later, patients present with global amnesia, slowing motor functions, with death typically occurring within 9 years after diagnosis [6]. The dysregulation in the metabolism of amyloid precursor protein (APP) and consequent deposition of amyloid-β (Abeta) peptide (derived from the APP by proteolytic degradation involving the specific proteases β- and γ-secretase, acting at the *N*- and *C*-terminal cleavage site, respectively) has been envisaged as crucial for the development of neurodegeneration in AD [7–9]; key features include the deposition of the Abeta in the form of senile (or amyloid) plaques, the formation of neurofibrillary tangles, and the loss of neurons and synapses in specific brain regions [10]. Abeta deposition begins 10–20 years before the appearance of clinical dementia. During this time, the brain is confronted with increasing amounts of toxic Abeta peptides and both the innate and the adaptive immune systems may play an important role in the disorder. Innate immunity in the brain is mainly represented by microglial cells (innate immune cells of bone marrow-derived monocytic cells which infiltrate into the brain contributing to the development of microglial reaction in AD) [11,12], which phagocytose and degrade Abeta. As the catabolism of Abeta decreases, glial cells become overstimulated and damage or kill neurons by releasing inflammatory (neurotoxic) molecules such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, chemokines [IL-8, macrophage inflammatory protein (MIP)-1α, monocyte chemo-attractant protein-1], nitric oxide (NO) or reactive oxygen metabolites (ROMs) [1,7,13,14]. In this respect, glial activation and expression of cytokines may act in synergy with other genetic and acquired environmental risks culminating in the development of this immune-mediated disease involving defective immune regulation and autoimmunity [15–18].

Specifically, microglia as well as astrocytes, macrophages and dendritic cells [(DCs), the most potent antigen-presenting cells (APCs)], are the immune effector cells in the central nervous system (CNS) concomitantly with inflammatory brain disease and play a significant role in the host defense against invading agents including microorganisms [19,20]. Brain inflammation, characterized by reactive microglia and astrocytes, is noticed in close vicinity of amyloid plaques in AD and in transgenic mouse models of the disease [11,21]. Microglia contain several antigenic and functional markers similar to DCs and macrophages [22] and can function as main APCs within the CNS [23,24]; microglia express immune-related antigens, constitutively HLA-DR *in situ*, the DC marker RFD1 upon activation [23,24], and, comparable to DCs [11], may also present antigen in the context of major histocompatibility complex (MHC) to CD4+ and CD8+ T lymphocytes (TLs). In this regard, MHC class II (HLA-DR) antigen-positive reactive microglia were observed throughout the cortex of post-mortem brains of patients with AD, particularly concentrated in the areas of senile plaque formation [25], and enriched microglial cultures alone were capable of stimulating TL responses or the CD4+ TL subset, a response which could be inhibited by an anti-MHC class II blocking antibody [26]. The interaction of activated CD4+ T cells with microglia led to a pro-inflammatory T helper type 1 (Th1) response, with a Th1 type cytokine expression profile involved in the pathogenesis of AD [11] via apoptosis, representing an important contributor to induction, progression and pathology of neurodegeneration in AD [27–30]. Th1 response, by secreting substantial levels of pro-inflammatory Th1 type cytokine TNF-α [11,31] leads to TNF-α-related apoptotic neuronal cell death in neurodegenerative diseases including AD [30,32]; TNF death receptor pathway and caspases are activated in the early stages of neuronal degeneration in AD [33]. Moreover, the Fas–Fas ligand (FasL) pathways may contribute to mechanisms of neuronal loss and neuritic degeneration in AD [34]. Hyperexpression of Fas mRNA and surface Fas receptor on TLs may explain the occurrence of inflammatory cellular infiltrates in the CNS of AD patients [35] leading to apoptotic damage; the key apoptosis regulator FasL may participate in both neuronal and immune cell apoptosis in AD [36]. A CD8+ cell-mediated apoptotic mechanism (activated cytotoxic TLs) may also play a pathogenic role in AD [17]. Additional recent studies have identified novel pathways, including the Wnt pathway and the serine–threonine kinase Akt, as central modulators that oversee cellular apoptosis in AD and the formation of neurofibrillary tangles through their downstream substrates including Bad, and Bcl-xL, and glycogen synthase kinase-3β [29]. Besides, Abeta directly induces neuronal apoptotic death (involving JNK activation, Bcl-w downregulation, and release of mitochondrial Smac), suggesting a role of Abeta neurotoxicity in AD neurodegeneration, responsible in part for the cognitive decline found in AD patients [37,38]. Although there is evidence suggesting a role of autoreactive Abeta-specific TLs in the elimination of this peptide, this beneficial mechanism seems to be impaired in the majority of patients with AD [39], thereby escalating the detrimental effect of

TLs in AD [40]. Notably, apoptotic, rather than necrotic, microglia associated nerve cell death appears as likely to underlie a number of common neurological conditions including AD, Parkinson's disease, glaucoma (defined as ocular AD) or multiple sclerosis [41–45]. The latter disease, for example, is also crucially dependent on activation of pro-inflammatory Th1-Ls by APCs, resistance of TLs 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 [11,46]. Summarizing, the above-mentioned data describe the current evidence for cellular immune defective and apoptotic mechanisms playing an important role in the neurodegenerative process in AD.

Regarding humoral immunity, recent evidence suggests that the possible presence of anti-neuronal antibodies and autoimmune mechanisms may be responsible for eliciting neuronal cell death in AD [47]. A key finding not only demonstrated the abnormal presence of anti-brain autoantibodies [48,49] and human immunoglobulins (Ig) [47,50] in the brain parenchyma of AD tissues, but, most significantly, 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 the 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 [47,50]. The evidence that autoantibodies may contribute to neuronal cell death in AD is also consistent with a wider literature in medicine implicating a causative role for autoantibodies in many peripheral neuropathies including Guillain-Barré syndrome [51] that share pathogenetic similarities with AD as well as glaucomatous optic neuropathy [52]. The autoantibodies directed toward retinal antigens may be involved in facilitating apoptotic cell death in glaucoma patients [53]. Therefore, apart from cellular immunity, abnormalities of humoral immunity appear to play a role in the pathogenesis of AD.

The early events underlying AD remain uncertain, although environmental factors may be involved. In this respect, the possibility that microorganisms can cause AD has recently been addressed [3,54]; infiltration of the brain by pathogens acts as a trigger or co-factor for AD, with *Herpes simplex* virus type 1 and *Chlamydophila* being implicated most frequently [3,55]. These pathogens may cause the neurological damage that results in AD by eliciting inflammation. 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 [3].

Helicobacter pylori

Since its original description by Warren and Marshall [56], *H. pylori*, a curved spiral gram-negative bacterium that colonizes the gastric mucosa of most humans worldwide (more than one half of the world's population is infected with this bacterium, mainly affecting older adults in the developed world) has been linked with a number of upper digestive diseases, particularly peptic ulcer disease that was viewed, like AD, as a classic degenerative condition, resulting from some toxic combination of *H. pylori* and stress, chemical irritants and bad genes [54,57,58]. Moreover, this bacterium has been associated with extradigestive disorders [51,59–61] such as functional vascular disorders caused by vascular dysregulation, atherosclerosis [62], hypertension, cardiovascular and/or cerebrovascular ischemia, and stroke [63], all of which have been found to be risk factors for AD, mainly by impairing blood–brain barrier, a common denominator associated with various degrees of dementia including AD [47,64–67]; these conditions contribute to the clinical manifestations and worsening of AD [68].

As in the case of AD, comparable cellular immune-mediated and apoptotic pathogenic features can also be introduced for *H. pylori* infection. Although *H. pylori* does not invade the gastric lamina propria, it induces an infiltrate of granulocytes and TLs that plays a major role in the pathology of upper gastrointestinal diseases. Specific subsets of infiltrating TLs also play a central role in controlling the outcome of this pathogen via the cytokine response induced by the *H. pylori* infection [69,70]; while the type of host immune response against *H. pylori* is crucial for the outcome of the infection, this response does not enable the immune system to clear the infection and may instead be detrimental to tissue integrity [70,71]. This bac-

terium elicits a complex immune response, involving both the innate and the adaptive immune responses. The dense infiltration of the gastric mucosa with cells of the immune system suggests that a complex interplay between APCs and other immune cells may be important for the development of *H. pylori*-induced gastric pathologies [72]. The immune response is triggered by presentation of antigen peptides on the major histocompatibility assembly of the APCs with the assistance of costimulatory molecules such as B7-1 (CD80) and B7-2 (CD86) [73]. Considering that DCs are professional APCs initiating T-cell responses and important mediators between the innate and cognate immune system [69,74], the initial immune response toward bacteria is characteristically dominated by DCs and other APCs; lamina propria macrophages also act as APC in the *H. pylori*-infected gastric mucosa [73]. Therefore, DC activation by *H. pylori* is essential for the development of an immune response [69], and stimulation/maturation of DCs are characteristically associated with the expression of bacterial epitopes on MHC on the surface together with costimulatory molecules [75]. *H. pylori* infection upregulates the expression of MHC class II antigens on gastric epithelium [76,77]; gastric epithelial cells may acquire APC properties in *H. pylori* infection by *de novo* expression of HLA-DR and costimulatory molecules [76]. Moreover, *H. pylori* induces DC activation, maturation as well as antigen presentation; its outer membrane proteins are capable of activating DCs, and DCs pulsed with *H. pylori* were shown to induce Th1 effector responses [69,74,75]. In this regard, *H. pylori* and its secreted products contribute to TL recruitment to the gastric mucosa and the responding TLs have an activated memory Th1 phenotype [70]. Therefore, *H. pylori*-associated gastroduodenal pathologies can be regarded as a Th1-driven immunopathological response to a number of *H. pylori* antigens. Specifically, in *H. pylori*-related autoimmune gastritis, cytolytic TLs infiltrating the gastric mucosa cross-recognize different epitopes of *H. pylori* proteins and gastric H^+/K^+-ATPase autoantigen (a significant proportion of the CD4+ TL clones proliferated in response to H^+/K^+-ATPase showing a Th1 profile) and this bacterium may lead to gastric autoimmunity via molecular mimicry [71,78]; activation of gastric H^+/K^+-ATPase-specific Th1 TLs is critical in the pathogenesis of gastric autoimmunity and atrophy in humans [78]. A predominant *H. pylori*-specific Th1 response is characterized by a high TNF-α, interferon (IFN)-γ, IL-2 and IL-12 production [71,72] leading to gastric epithelial cell apoptotic damage. Several studies reported that the Fas–FasL system is involved in *H. pylori*-induced apoptosis, and T cell-mediated cytotoxicity via Fas–FasL signaling may contribute to the induction of apoptosis in gastric epithelial cells during *H. pylori* infection [71,72]. Inflammatory cytokines present during *H. pylori* infection, such as IFN-γ, enhance activation of the Fas signaling pathway *in vitro* [79]. In this context, Fas and TNF-α receptor-1 expressed on gastric epithelial cells from *H. pylori*-infected patients are responsible for the accelerated cell apoptosis [80,81]; TNF-α induces the apoptotic death of gastric parietal cells contributing to the atrophy and hypochlorhydria of the gastric mucosa noticed during chronic *H. pylori* infection [82]. Additional evidence indicates that *H. pylori* is capable of inducing apoptotic effects through the mitochondrial apoptotic pathway involving activation of the proapoptotic proteins Bax and Bak, activation of certain caspases or through inducible NO [83–88]; NO is a rapidly diffusing gas and a potent neurotoxin that may contribute to the apoptotic neuronal cell death in degenerative neuropathies including AD [89] and glaucomatous optic neuropathy [90].

The above-mentioned data describe the evidence for the irregular cellular immune and apoptotic mechanisms playing an important role in the *H. pylori*-associated gastrointestinal pathologies and potentially affecting the neurodegenerative process in AD. In this respect, *H. pylori* infection induces cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves [51], thereby contributing to potential neural tissue damage in AD.

Comparable data to AD abnormalities of humoral immunity can also be considered for *H. pylori* infection. Indeed, gastric autoimmunity is well established in patients with *H. pylori* infection associated with induction of autoantibodies that cross-react with the gastric mucosa [91]. This type of autoreactivity is linked with the presence and degree of inflammation and atrophy of the glands [92]. Moreover, serum parietal cell autoantibodies correlate with anti-*H. pylori* antibody titers [93]. Therefore, the serological titer of anti-*H. pylori* seems to reflect the autoimmunity status that correlates with gastric mucosal atrophy, thereby indirectly offering evidence for the severity of the histological inflammatory changes [94]. Interestingly, molecular mimicry of host structures by the saccharide portion of lipopolysaccharides of the gastrointestinal pathogens *Campylobacter jejuni* (*C. jejuni*) and *H. pylori* is thought to be connected with the development of autoimmune sequelae observed in neuropathies. *C. jejuni*, a principal cause

of gastroenteritis, is the most common antecedent infection in Guillain-Barré syndrome, an inflammatory autoimmune neuropathy. 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 Guillain-Barré syndrome, but are generally absent in enteritis cases. Collective data suggest that the antibodies are induced by the antecedent infection with *C. jejuni*, and subsequently react with nerve tissue causing its damage [95], possibly by apoptosis. In addition, several IgG antibodies against *H. pylori* proteins are found in the cerebrospinal fluid in 57% of patients with Guillain-Barré syndrome. No cross-reactivity against *C. jejuni* is observed and these antibodies may also be involved in the immune responses of patients with Guillain-Barré syndrome [96]. Similarly, 46% of patients with Guillain-Barré syndrome have specific IgG antibodies to VacA of *H. pylori* in the cerebrospinal fluid, 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 resulting in demyelination in some patients within the cerebrospinal fluid [97]. In this context, it is relevant to speculate that such anti-*H. pylori*-mediated apoptotic mechanisms might also lead to degeneration of ganglion cells in AD neuropathy or other degenerative neuropathies such as achalasia [98] or glaucoma [45]. Support for this theory is provided by our recent observations indicating that the titer of anti-*H. pylori* IgG antibodies in the aqueous humor of patients with glaucoma may reflect the severity of glaucomatous damage [99]. Summarizing, the above-mentioned data describe the current evidence for abnormalities of humoral immunity playing a significant role in the *H. pylori*-associated gastric pathologies and potentially contributing to other degenerative neuropathies including AD neuropathy.

Relationship between *H. pylori* infection and AD (Fig. 1)

Although degenerative diseases of the CNS, including AD, have an increasingly high impact in aged population, their association with *H. pylori* infection has not as yet been thoroughly researched. This issue has only recently been addressed by two studies [100,101]. A higher seropositivity for anti-*H. pylori* IgG antibodies was reported in 30 patients with AD than in 30 age-matched controls [102]. However, this serological test has limitations because it does not discriminate between current and old infections [102]. Such a distinction is essential because current *H. pylori* infection, as mentioned, induces humoral and cellular immune responses that, owing to the sharing of homologous

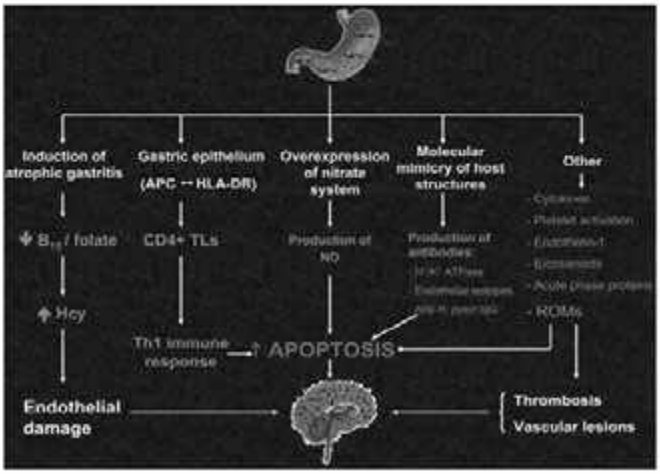

Figure 1 Schematic presentation of the proposed pathophysiological mechanisms by which *Helicobacter pylori* infection might contribute to Alzheimer's disease. (Hcy, homocysteine; TLs, T lymphocytes; APC, antigen-presenting cells; NO, nitric oxide; ROMs, reactive oxygen metabolites).

epitopes (molecular mimicry), cross-react with components of nerves [51], thereby affecting or perpetuating neural tissue damage. Moreover, eradication of *H. pylori* infection might delay AD progression, particularly at early disease stages. Based on the histological analysis of gastric mucosa biopsy for the documentation of *H. pylori* infection, we investigated whether *H. pylori* infection is associated with AD by introducing the histological method established as the actual gold standard for diagnosis of *H. pylori* infection [103]. In our cohort of Greek patients, 88% of the AD patients exhibited histologically proven *H. pylori* infection, whereas the rate of infection was significantly lower in the anemic control group (46.7%) (Table 1) [101]. Moreover, multifocal chronic gastritis (body and antrum atrophy) was observed in the vast majority of our patients compared with controls (Table 2). These patterns of *H. pylori*-related chronic gastritis have also been reported by others [104]. In addition, an increased serum homocysteine (Hcy) concentration was observed in our AD patients, a finding also reported by others [100]. Chronic gastritis, as a result of *H. pylori* infection, 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 [100,105]. The elevated Hcy, in turn, could trigger endothelial damage and result in atherothrombotic disorders and AD. In this respect, investigators reported that *H. pylori*-induced chronic atrophic gastritis or atrophic gastritis *per se* decreases serum vitamin B12 and folate concentrations, thereby increasing the Hcy, a potent contributor to vascular disorders; serum Hcy concentrations correlated inversely with serum vitamin B12 and folate levels and positively with atrophic scores [105]. Hcy appears to be an independent risk factor for dementia and AD as well as for vascular disease; it is thought to be implicated in endothelial damage and neurodegeneration via oxidative injury in these diseases [106]. Besides, it has been shown that the serum Hcy concentration correlates with the severity of dementia, and is a significant predictor of the severity of dementia [107]. Considering the above-mentioned data, we can speculate that *H. pylori* infection might contribute, at least in part, to the pathogenesis of AD through induction of chronic atrophic gastritis and Hcy sequence. However, further studies with large number of patients are needed to elucidate this field.

Notably, the present study did not establish causality, because this requires showing that eradication of *H. pylori* alters the course of AD. The *H. pylori* and AD association, reported herein, may be explained by an existence of a common (genetic?) factor that predisposes to both *H. pylori* infection and AD. An outgrowth of this alternative possibility would be that eradicating *H. pylori* infection would not necessarily have any effect on the development or progression of AD. Genetic

Table 2 Endoscopic evidence of esophagitis, gastritis, duodenitis, inefficiency of lower esophageal sphincter (LES), and peptic ulcer disease in patients with Alzheimer's disease (AD) and anemic control participants

Characteristic	AD patients ($n = 50$)	Anemic controls ($n = 30$)	*P* value
Normal	0 (0%)	6 (13%)	0.003
Esophagitis	3 (6%)	2 (6.7%)	NS
Multifocal gastritis	49 (98%)	21 (70%)	<0.001
Duodenitis	30 (60%)	13 (43.3%)	NS
LES inefficiency	38 (76%)	8 (26.7%)	<0.001
Peptic ulcer disease	6 (12%)	0 (0%)	0.04

Table 1 *Helicobacter pylori* positivity, and total homocysteine (Hcy) concentrations in patients with Alzheimer's disease (AD) and anemic controls

Characteristic	Patients with AD ($n = 50$)	Anemic controls ($n = 30$)	Odds ratio (95% CI)	*P* value
Mean ± SD age (range) y	65.0 ± 6.9 (53–80)	62.2 ± 8.6 (44–70)	–	0.07
No. of men/No. of women	18/32	14/16	–	0.48
Positive urease test (gastric mucosa)	30 (60%)	14 (46.7%)	1.7 (0.7–4.3)	0.35
Mean serum anti-*H. pylori* IgG concentration (U/mL)	34.0 ± 40.1	17.0 ± 18.1	–	0.016
Anti-*H. pylori* IgG > 10 U/mL	31 (62%)	14 (46.7%)	1.9 (0.7–4.7)	0.24
Histologically confirmed presence of *H. pylori*	44 (88%)	14 (46.7%)	8.4 (2.4–28.7)	<0.001
Mean serum total Hcy concentration (μmol/L)	17.7 ± 4.9	13.5 ± 4.0	–	0.001

susceptibility to *H. pylori* infection has been reported in studies of monozygotic twins [104]. Genetic transmission in an autosomal dominant fashion has also been reported in AD [108]. However, future investigation for any potential detection of common genetic alterations causing susceptibility to both conditions is needed to elucidate this hypothesis.

Closing remarks

H. pylori infection may influence the pathophysiology of AD by one of the following mechanisms (summarized in Fig. 1): (1) promoting platelet and platelet–leukocyte aggregation [109]. Platelet activation and aggregation have also been proposed to play pathophysiologic roles in the development of AD [110]; platelets are a source of the major constituent of senile plaques Abeta, considered to be the primary and central event in the etiology and pathogenesis of AD, and both increased platelet activation and increased circulating Abeta have been identified in AD. Moreover, Abeta role may be to inflict vascular damage, and hence impair blood–brain barrier function; increased blood–brain barrier permeability, increased platelet aggregation and cerebral vasoconstriction predispose the AD brain to thrombotic and/or ischemic events [47,111,112], (2) inducing chronic atrophic gastritis with concomitant decrease in vitamin B12 and folate concentrations, thereby increasing the Hcy, an independent risk factor for AD and a potent contributor to vascular disorders implicated in endothelial damage and neurodegeneration via oxidative injury, (3) releasing large amounts of pro-inflammatory and vasoactive substances, such as cytokines (IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-γ), eicosanoids (leukotrienes, prostaglandins catalyzed by cyclo-oxygenase enzymes), and acute phase proteins (fibrinogen, C-reactive protein) [61,113] involved in a number of vascular disorders, including AD [32,114–120] and other AD-related neuropathies such as glaucoma [45], (4) stimulating mononuclear cells to produce a tissue factor-like procoagulant that converts fibrinogen into fibrin [121], (5) causing the development of cross-mimicry between endothelial and *H. pylori* antigens, (6) producing ROMs and circulating lipid peroxides [59,122] that have also been involved in the pathophysiology of AD [4]; 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 [123]. ROMs accumulation impairs endothelial barrier function and promotes leukocyte adhesion, induces alterations in normal vascular function and results in the development of AD [124], events that are also triggered in *H. pylori*-induced gastrointestinal injury [125]. Moreover, there is evidence for a role of oxidative damage in contributing to Abeta deposition in AD [126] and (7) influencing the apoptotic process that may also be an important form of cell death in many neurodegenerative diseases including AD, glaucomatous neuropathy [28,45,99,127], or Down syndrome that predisposes to the early onset of the neurodegeneration of AD [116]. In particular, increased endothelin-1 (a potent constrictor of arterioles and venules), NO, and inducible nitric oxide synthase (iNOS) levels are associated with *H. pylori* infection [128,129]. Relative data in AD indicate that endothelin-1-like immunoreactivity in the AD brains is significantly more increased in frontal and occipital cortex than those in the control brains, thereby explaining the decreased cerebral blood flow in AD patients [130]. The immunoreactive cells are often located in small clusters close to blood vessels [131], and Abeta peptides potentiate endothelin-1-induced vasoconstriction [132]. Besides, recent evidence in humans indicates that the expression of nitrergic system, the synthesis of NO, the peroxynitrite reactive production and the protein tyrosine nitration are activated over the entire chronic course of AD, and that the presence of Abeta increases the presence of neuronal NOS and iNOS isoforms over the chronic course of AD in pyramidal-like neurons [89]; the overproduction of NO, the increase in both peroxynitrite and superoxide production, the mitochondrial membrane depolarization and the caspase activation contribute to neuronal death [89,123], mainly via apoptosis. Further supporting this concern, it has been shown that endothelin-1-induced vasoconstriction of the anterior optic nerve vessels and NO modulation of vascular tone in the ophthalmic artery may produce glaucomatous damage [133]. Evidence of NO neurotoxicity in glaucoma and probably in AD neuropathy [89,90] is also provided by experimental evidence demonstrating that retinal ganglion cell apoptosis is attenuated by selective inhibitors of iNOS or neutralizing antibodies against TNF-α, thereby suggesting that inhibition of the inducible isoform NOS2 or TNF-α may provide novel therapeutic targets for neuroprotection in the treatment of glaucomatous optic neuropathy [89]; because TNF-α induces apoptotic neuronal cell death in neurodegenerative diseases including AD [30,33,134], further studies are needed to clarify if comparable inhibitions of TNF-α may also provide novel therapeutic targets for neuroprotection in the treatment of AD.

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Association between *Helicobacter pylori* infection and mild cognitive impairment

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The association of *Helicobacter pylori* infection and Alzheimer's disease (AD) has recently been addressed, but no relative data exist regarding mild cognitive impairment (MCI), a prodromal phase of AD. The aim of this prospective study was to evaluate *H. pylori* infection, by histology in a Greek MCI cohort. Sixty-three consecutive patients with amnestic MCI and 35 normal controls underwent upper gastrointestinal endoscopy, histologic and serological examinations. The prevalence of *H. pylori* infection was 88.9% (56/63) in MCI patients and 48.6% (17/35) in anaemic controls, as confirmed by biopsy ($P < 0.001$, odds ratio: 8.47, 95% CI: 3.03–23.67). Mean serum anti-*H. pylori* IgG concentration and plasma total homocysteine (Hcy) titre were higher in MCI patients than controls (74.86 ± 57.22 vs. 17.37 ± 9.30 U/ml; and 16.03 ± 4.28 vs. 13.5 ± 1.20 μmol/l; $P < 0.001$ and $P = 0.015$, respectively). When compared with the anaemic participants, MCI patients exhibited more often multifocal (body and antral) gastritis (92.1% vs. 68.6%; $P = 0.03$); in *H. pylori* positive MCI patients cognitive state correlated with serum anti-*H. pylori* IgG concentration. In conclusion, *H. pylori* prevalence was significantly higher in MCI patients than controls. This infection might contribute, at least in part, to the pathophysiology of MCI, possibly through induction of chronic atrophic gastritis and elevated Hcy consequences.

Introduction

Mild cognitive impairment (MCI) refers to memory deficits in excess of normal ageing, but not sufficient for the diagnosis of Alzheimer's disease (AD). Particular recent interest in MCI arises from the fact that MCI appears to be a prodromal phase and therefore highly predictive of subsequent AD [1]. MCI is a heterogeneous clinical entity with multiple sources of heterogeneity; identifying aetiopathogenic subgroups of patients at a high risk for progression to AD dementia and establishing more specific therapeutic strategies optimally effective at an early stage [2] may delay the onset or prevent AD dementia.

Although the early events underlying MCI and AD remain uncertain, the consideration that microorganisms can cause AD or possibly MCI has recently been addressed [3,4]: infiltration of the brain by pathogens acts as a trigger or co-factor for AD, with *Herpes simplex* virus type 1 and *Chlamydophila* being implicated most frequently [3]. These pathogens may cause the neurological damage that results in AD by eliciting inflammation.

In this respect, the association of *Helicobacter pylori* infection and AD has also only recently been addressed by two studies [5,6]. A higher seropositivity for anti-*H. pylori* 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 [7]. Such a distinction is essential because current *H. pylori* infection induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves [7,8], thereby affecting or perpetuating neural tissue damage. Moreover, eradication of *H. pylori* infection might delay AD progression, particularly at early disease stages including MCI. Based on the histologic analysis of gastric mucosa biopsy for the documentation of *H. pylori* infection, we [6] reported a higher prevalence of *H. pylori* in patients with AD than in age-matched controls, accompanied with increased homocysteine (Hcy) concentration, an independent risk factor for dementia and AD, thereby suggesting an association between these two diseases [6].

Because, to our knowledge, no attempt has been made until now to investigate the prevalence of

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H. pylori infection in MCI patients, the objective of this study was to evaluate the histologic analysis of gastric mucosa biopsy for the documentation of current *H. pylori* infection in a Greek cohort of amnestic MCI patients.

Patients and methods

We studied 65 consecutive patients referred to the Memory and Dementia Outpatient Clinic, who fulfilled the diagnostic criteria for amnestic MCI, single (a-MCI) or multiple domain (md-MCI + a). Two patients refused to undergo upper gastrointestinal (GI) endoscopy and they were excluded from the study. Therefore, the remaining 63 patients (41 females; mean age of 66.32 ± 7.96 years, range 50–78 years) (Group A) were included in the study. Screening procedure for their evaluation was conducted at their first visit to the Memory and Dementia Outpatient Clinic. a-MCI criteria, described by Petersen, include five points: (i) memory complaint usually corroborated by an informant, (ii) objective memory impairment for age, (iii) essentially preserved general cognitive function, (iv) largely intact functional activities and (v) not demented. Diagnosis of md-MCI + a required additional impairment at least in one cognitive domain such as language, executive function and visuospatial skills [9]. Insidious onset and gradual progression underlined the neurodegenerative nature of the procedure. All patients fulfilled a screening battery that included Mini-Mental State Examination (MMSE, cutoff = 24), Cambridge Cognitive Test (CAMCOG, cutoff = 80), Functional Rating Scale for the Severity of Dementia (FRSSD, cutoff = 5), Functional Cognitive Assessment Scale (FUCAS, cutoff = 42), Neuropsychiatric Inventory (NPI), Geriatric Depression Scale, 15-item (GDS, cutoff = 5) and Hamilton Depression Rating Scale (HDRS, cutoff = 11). The Petersen a-MCI criteria were operationalized as (i) impaired memory: Word List Delayed Recall score of −1 SD below mean; (ii) normal mental status: MMSE score of > 24; (iii) normal daily functioning: FRSSD score < 5 and FUCAS score < 42; (iv) memory complaint: subjective response to standardized question; (v) not demented: Clinical Dementia Rating Scale score 0 or 0.5 [10].

After inclusion patients fulfilled a second neuropsychological battery that included Immediate Recall Rey Auditory Verbal Learning Task, Free Delayed Recall Rey Auditory Verbal Learning Task, Verbal Fluency (number of animals in 1 and 2 min), Boston Naming Test 60-item, Stroop cards 1–3, Trail Making Test A and B, Symbol Digit Substitution Test, Copy Rey-Figure, Delayed Recall Rey Figure and Clock Drawing Test. Impaired performance in at least one of the above-mentioned tests apart from Immediate Recall Rey Auditory Verbal Learning Task and Free Delayed Recall Rey Auditory Verbal Learning Task, indicated md-MCI + a.

Clinical assessments were based on a standardized format consisting of a neurological and physical examination, an MRI of the brain, and blood chemistries to exclude other metabolic causes for cognitive decline. Patients were excluded if they had taken cholinesterase inhibitors, memantine, or any other pharmacological treatment for dementia, histamine$_2$-receptor antagonists, proton pump inhibitors, antibiotics, bismuth compounds, or non-steroidal anti-inflammatory drugs (excluding low doses of aspirin) in the preceding 4 weeks. Patients were also excluded if they had known depression or thyroid disorders; undergone previous gastric surgery; received anticoagulant therapy; were alcohol abusers; were allergic to penicillin or macrolides; had gastric cancer or other neoplasms; or had severe cardiac, pulmonary, kidney, or liver disease.

Control subjects (Group B) consisted of 35 patients without MCI (18 females, mean age of 68.22 ± 8.45 years, range 50–84 years), who underwent upper GI endoscopy for investigation of mild iron deficiency anaemia but in whom endoscopy did not reveal any obvious finding. None of the subjects received any treatment like ferrous sulphate before the diagnosis. Neuropsychological assessment of control subjects included MMSE, CAMCOG, FRSSD and GDS scales. Concomitant medical diseases are described in Table 1.

All patients (63 MCI and 35 control patients) underwent diagnostic upper GI endoscopy after informed consent.

The study was designed according to the principles of the Declaration of Helsinki (1964) and the study protocol was approved by the local ethics committee.

Table 1 Concomitant medical conditions in MCI patients and control subjects

	MCI ($n = 63$)	Anaemic controls ($n = 35$)	P value
Hypertension	20	9	0.531
Diabetes mellitus type II	2	1	0.930
Osteoporosis	3	2	0.837
Spondyloarthritis	3	2	0.837
Rheumatoid arthritis	1	0	0.484
Irritable bowel syndrome	4	3	0.682
Coronary heart disease	2	1	0.930
Open-angle glaucoma	1	0	0.484

Study design

Helicobacter pylori detection methods were reported previously [6,7,11]. Biopsy urease test and histopathology process were also described previously [6,7,11].

Statistics

For comparison of the age (years) between MCI and control subjects the independent samples *t*-test was used, whereas for gender the chi-square test with Yates' correction was applied. The latter test was also used to compare the prevalence of *H. pylori* infection between MCI and control subjects. Odds ratios and 95% confidence intervals (CI) were also calculated between these two groups. A two-tailed *t*-test was used for comparison of the mean serum anti-*H. pylori* IgG and the mean serum total Hcy concentrations between the two study groups. Significance was set at $P < 0.05$.

Results

Mean age and gender ratios did not differ between MCI and anaemic control participants (Table 2). In the MCI patients the mean MMSE score was 27.62 ± 1.93, the mean CAMCOG score was 95 ± 5.4 and the mean FRSSD score was 4.2 ± 1.05. In the anaemic participants mean MMSE score was 29.49 ± 0.51, the mean CAMCOG score was 97.11 ± 4.43 and the mean FRSSD score was 1.17 ± 1.04, respectively.

The prevalence of *H. pylori* infection was 88.9% (56/63) in the MCI patients and 48.6% (17/35) in the anaemic control patients, as confirmed by the presence of *H. pylori* bacteria histologically (χ^2: 19.25, $P < 0.001$, odds ratio: 8.47, 95% CI: 3.03–23.67). As shown in Table 2, *H. pylori* was detected in the gastric mucosa (antrum, corpus, or both) in 58.7% (37/63) of MCI patients and in 42.9% (15/35) of the anaemic control group (P = NS) by urease test. When compared with the control values the mean serum anti-*H. pylori* IgG concentration was significantly higher in MCI patients (74.86 ± 57.22 vs. 17.37 ± 9.30 U/ml; $P < 0.001$). Mean plasma total Hcy titre was also significantly higher in MCI patients than in controls (16.03 + 4.28 vs. 13.50 + 1.20 μmol/l, $P = 0.015$).

Table 3 shows the endoscopic evidence of oesophagitis, gastritis, duodenitis, inefficiency of lower oesophageal sphincter, and peptic ulcer disease noted in MCI patients and anaemic controls. When compared with the anaemic participants, MCI patients exhibited less often normal appearance of the gastric mucosa ($P = 0.042$) and more often multifocal (body and antral) gastritis ($P = 0.03$) or lower oesophageal sphincter inefficiency ($P < 0.001$). Comparisons for endoscopic evidence of oesophagitis and duodenitis or peptic ulcer disease did not reveal any statistical differences between MCI patients and anaemic control participants. Oesophagitis, multifocal gastritis and peptic ulcer disease were confirmed histologically.

The histologic grading of *H. pylori* infection (according to the Sydney system), in MCI patients versus the anaemic controls, included atrophy grade, chronicity, activity and intestinal metaplasia on a scale of 0 (absent) to 3 (high). Grade 3 was noted in 7 of 63 (11.1%) MCI patients and in none of the anaemic control participants ($P = 0.041$) whereas grades 0, 1 and 2 did not differ significantly between the MCI patients and the anaemic control participants.

In the subgroups of *H. pylori*-positive MCI patients ($n = 56$) and controls ($n = 17$), anti-*H. pylori* IgG

Table 3 Endoscopic and histologic evidence of oesophagitis, gastritis, duodenitis, inefficiency of lower oesophageal sphincter (LOS), and peptic ulcer disease in patients with MCI and anaemic controls

Characteristics	MCI patients ($n = 63$)	Anaemic controls ($n = 35$)	*P* value
Normal	3 (4.8)	6 (17.1)	0.042
Oesophagitis	3 (4.8)	2 (5.7)	0.830
Multifocal gastritis	58 (92.1)	24 (68.6)	0.030
Duodenitis	29 (46.0)	15 (42.9)	0.765
LOS inefficiency	54 (85.7)	12 (34.3)	<0.001
Peptic ulcer disease	4 (6.3)	0 (0)	0.133

Values are given as *n* (%).

Table 2 *Helicobacter pylori* positivity in patients with MCI and anaemic controls

Characteristics	MCI patients ($n = 63$)	Anaemic controls ($n = 35$)	Odds ratio (95% CI)	*P* value
Age, mean (SD), years	66.32 (7.96)	68.22 (8.45)	NA	0.279
Women, *n* (%)	41 (65.1)	18 (51.4)	NA	0.186
Positive urease test (gastric mucosa), *n* (%)	37 (58.7)	15 (42.9)	1.90 (0.82–4.30)	0.13
Serum anti-*H. pylori* IgG concentration, mean (SD), U/ml	74.86 (57.22)	17.37 (9.30)	NA	<0.001
Anti-*H. pylori* IgG > 10 U/ml, *n* (%)	46 (73.0)	11 (31.4)	5.90 (2.38–14.50)	0.001
Histologically confirmed presence of *H. pylori*, *n* (%)	56 (88.9)	17 (48.6)	8.47 (3.03–23.67)	<0.001
Serum total Hcy concentration, mean (SD), μmol/l	16.03 (4.28)	13.5 (1.2)	NA	0.015

> 10 U/ml were more frequent in *H. pylori* positive MCI patients than in controls (45/56 vs. 9/17, $P = 0.024$). The mean serum anti-*H. pylori* IgG concentration was also higher in the *H. pylori*-positive subgroup of MCI patients (83 ± 52.1 U/ml vs. 23.6 ± 21.1 U/ml, $P < 0.001$). Grade 3 Sydney score tended to be more frequent in *H. pylori*-positive MCI patients, although this difference did not reach significant level (7/56 vs. 0/17, $P = 0.125$).

In the subgroup of *H. pylori*-positive MCI patients, a negative but not significant correlation was observed between MMSE scores and Sydney scores (Spearman's $R = -0.023$, $P = 0.876$), and between MMSE scores and anti-*H. pylori* IgG serum concentrations (Pearson's $R = -0.007$, $P = 0.964$). Concerning the association between CAMCOG score and disease markers in the subgroup of *H. pylori*-positive MCI patients, a significant negative correlation was found for anti-*H. pylori* IgG serum concentrations (Pearson's $R = -0.455$, $P = 0.008$) but not for Sydney scores (Spearman's $R = -0.014$, $P = 0.947$).

Discussion

Helicobacter pylori, a curved spiral Gram-negative bacterium that colonizes the gastric mucosa of more than half humans worldwide, has been associated with extradigestive disorders [7,11], such as atherosclerosis [12], hypertension [13], cardiovascular and/or cerebrovascular ischaemia and stroke [14], all of which have been found to be risk factors for MCI [1,15,16] and AD [5], by impairing mainly blood–brain barrier, a common denominator associated with various degrees of dementia, including MCI and AD [17,18]; these disorders [19] contribute to MCI conversion to clinical dementia, as well as the clinical manifestations and worsening of AD disease [1,5].

To the best of our knowledge, the present series suggests, for the first time, a possible link between *H. pylori* infection and amnestic MCI. In our cohort of Greek patients, 88.9% of the MCI patients exhibited histologically proven *H. pylori* infection whereas the rate of infection was significantly lower in the anaemic control group (48.6%). The prevalence observed in our MCI patients is quite similar to previously published figures in AD (88%) [6]. Moreover, the prevalence noticed in our control group is similar to other reported studies when using serodiagnostic assays to evaluate Greek and other cohorts (frequency distribution 34.1–61.6%) [20].

It is worth considering whether the rate of *H. pylori* infection in the control group has been negatively influenced by the coexistence of anaemia. There is no evidence to suggest that anaemia protects against development of *H. pylori* infection. Anaemic controls have been employed before [21], and the frequency of *H. pylori* infection in the anaemic control group matches that of the general population in Greece and that reported in other ethnic groups [20]. Furthermore, it is unlikely that individuals with iron-deficiency anaemia are protected against *H. pylori* infection because it is thought that the infection is actually associated with iron- and/or vitamin B_{12} deficiency anaemia [22]. In addition, eradication of *H. pylori* infection may be associated with reversal of iron and/or vitamin B_{12} deficiency and improvement of anaemia [23]. Besides, iron depletion to near-deficiency levels does not influence Hcy plasma concentrations [24], thus it seems unlikely that our control group with mild iron deficiency anaemia could be characterized by low Hcy levels.

Our series has relied on histologic analysis for the documentation of *H. pylori* infection. Although culture is the theoretic gold standard for detection of the bacterium, it has been shown that there is an excellent correlation with histologic identification. Therefore, for most studies, mucosal biopsy and histologic examination of the specimen for the presence of *H. pylori* and gastritis is the actual gold standard for diagnosis of *H. pylori* infection [7]. On the other hand, serology, urea breath test or urease diagnostic test (CLO test) would lead to limited results [7].

In this study, multifocal chronic gastritis (body and antrum atrophy) was observed in the majority (92%) of our patients compared with controls. These patterns of *H. pylori*-related chronic gastritis have also been reported by others [19]. Moreover, an increased serum Hcy concentration has been shown in our MCI patients, a finding reported by others in MCI and AD as well [5,25].

Chronic gastritis, owing to *H. pylori* infection, can lead to malabsorption of vitamins (B_{12}) and folate, which results in failure of methylation by 5-methyltetrahydrofolic acid and hence accumulation of Hcy [5,26,27]. Relative studies reported that *H. pylori*-induced chronic atrophic gastritis or atrophic gastritis *per se* decreases serum vitamin B_{12} and folate concentrations, thereby increasing the Hcy, a potent contributor to vascular disorders; serum Hcy concentrations correlated inversely with serum vitamin B_{12} and folate levels and positively with atrophic scores [26,27]. Hcy appears to be a risk factor for MCI dementia and AD as well as for vascular disease. It is thought to be implicated in endothelial damage and neurodegeneration via oxidative injury in these diseases [28,29]; oxidative damage has been described in the brain of subjects with MCI, suggesting that oxidative damage may be one of the earliest events in the onset and progression of AD [30]. Importantly, serum Hcy

concentrations are independently associated with the progression of MCI to AD and also correlate with the severity of dementia [25,31].

Considering the above-mentioned data, we can speculate that *H. pylori* infection, might contribute, at least in part, to the pathophysiology of MCI and AD through induction of chronic atrophic gastritis and Hcy consequences. Moreover, MCI patients with this pathogen accompanied by increased Hcy may be more likely to progress to AD. However, further studies with large number of patients are needed to elucidate these speculations.

Interestingly, the positivity status for *H. pylori* serology appeared to correlate with cognitive deterioration in our *H. pylori*-positive MCI patients. This correlation was present when CAMCOG scores were examined, whereas it was not significant when MMSE scores were examined, possibly due to the more wide range of CAMCOG values (90–105) and narrow range of MMSE values (24–29) in MCI patients. Of note, MMSE scale, as a sub-scale of CAMCOG, is a useful tool as only a crude screening procedure for dementia, whilst CAMCOG constitutes a more thorough evaluation of cognitive function. Therefore, it is reasonable to assume that CAMCOG, as a larger climax, would show a correlation even in our small sample size. Comparably, serum high levels of anti-*H. pylori* IgG antibodies also correlated with a more advanced clinical status and involvement of the proximal parts of peripheral nerves in patients with acute inflammatory demyelinating polyradiculoneuropathy (AIDP) [7]. Furthermore, the positivity status for *H. pylori* appeared to correlate with the severity of glaucomatous cupping in glaucoma patients [32]. It has been suggested that the increased titre of anti-*H. pylori* IgG antibodies observed in the serum samples of AIDP and glaucoma patients, may indirectly offer evidence for a role of this agent in the cascade events of the damage of the myelin and/or the axons of the peripheral nerves and glaucomatous optic neuropathy [7,32]. The most likely mechanism for the role of this organism is via molecular mimicry autoimmune sequelae. Future studies in large MCI cohorts throughout the clinical range are needed to support the hypothesis that the presence of IgG antibodies to *H. pylori* may also adversely influence progression of MCI neuropathy.

The present study did not establish causality, because this requires showing that eradication of *H. pylori* alters the course of MCI. The *H. pylori* and MCI association, reported herein, may be explained by an existence of a common (genetic?) factor that predispose to both *H. pylori* infection and MCI. An outgrowth of this alternative possibility would be that eradicating *H. pylori* infection would not necessarily have any effect on the development or progression of MCI. Genetic susceptibility to *H. pylori* infection has been reported in studies of monozygotic twins [19]. Familial MCI existence and genetic transmission in an autosomal dominant fashion in AD have also been reported [33]. However, future investigation for any potential detection of common genetic alterations causing susceptibility to both conditions is also needed to elucidate this hypothesis.

Helicobacter pylori infection may influence the pathophysiology of MCI–dementia–AD sequence by: (i) promoting platelet and platelet–leucocyte aggregation, also proposed to play pathophysiological roles in AD development [6,8]. Specifically, it has been reported altered patterns of amyloid precursor protein (APP) in platelets of patients with AD, playing a key role in the pathogenesis of the disease through the formation of Aβ peptide [34]. Similar finding was confirmed in MCI patients, suggesting that alteration of platelet APP forms is an early event in AD, and the measurement of these forms may be useful for the identification of preclinical AD in patients with MCI [35]; (ii) releasing proinflammatory and vasoactive substances involved in a number of vascular disorders including MCI, AD and other AD-related neuropathies such as glaucoma, defined as the 'ocular AD' [6,8,36,37]; (iii) stimulating mononuclear cells to produce a tissue factor-like procoagulant that converts fibrinogen into fibrin [6,8]; (iv) causing the development of cross mimicry between endothelial and *H. pylori* antigens; (v) increasing Hcy, a risk factor for MCI, dementia and AD, implicated in endothelial damage and neurodegeneration via oxidative injury in these diseases [6,25]; (vi) producing reactive oxygen metabolites and circulating lipid peroxides also involved in the pathophysiology of AD [6,8]; and (vii) influencing the apoptotic process, an important form of cell death in many neurodegenerative diseases including AD and possibly MCI [6,8,36]. Notably, current *H. pylori* infection appears to induce irregular humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves [7], thereby contributing and possibly perpetuating the apoptotic neural tissue damage observed in neurodegenerative diseases including AD and possibly MCI [8].

In conclusion, an association between *H. pylori* infection and MCI patients in a Greek cohort has been found. Whether this association is causal or coincidental needs confirmation by other rigorously controlled epidemiologic studies. If a causal link between *H. pylori* infection and progression from MCI in dementia is confirmed in the future, this may have a major impact on the pathophysiology and management of MCI–AD–dementia sequence.

Competing interests

None declared.

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RAPID COMMUNICATION

Evidence for the role of gastric mucosa at the secretion of soluble triggering receptor expressed on myeloid cells (strem-1) in peptic ulcer disease

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Abstract

AIM: To investigate the role of gastric mucosa at the secretion of sTREM-1 in peptic ulcer.

METHODS: Seventy two patients were enrolled; 35 with duodenal, 21 with gastric ulcer and 16 with chronic gastritis. Patients were endoscoped and gastric juice was aspirated. Patients with duodenal and gastric ulcer underwent a second endoscopy post-treatment. Biopsies were incubated in the absence/presence of endotoxins or gastric juice. Supernatants were collected and sTREM-1 and TNFα were measured by enzyme immunoabsorbent assays. Scoring of gastritis was performed before and after treatment according to updated Sydney score.

RESULTS: Patients with duodenal and gastric ulcer and those with chronic gastritis had similar scores of gastritis. sTREM-1 was higher in supernatants of tissue samples of *H pylori*-positive than of *H pylori*-negative patients with gastric ulcer. Median (± SE) sTREM-1 was found increased in supernatants of patients with gastric ulcer before treatment (203.21 ± 88.91 pg/1000 cells) compared to supernatants either from the same patients post-treatment (8.23 ± 5.79 pg/1000 cells) or from patients with chronic gastritis (6.21 ± 0.71 pg/1000 cells) ($P < 0.001$ and < 0.001, respectively). Similar differences for sTREM-1 were recorded among LPS-stimulated tissue samples of patients ($P = 0.001$). Similar differences were not found for TNFα. Positive correlations were found between sTREM-1 of supernatants from patients with both duodenal and gastric ulcer before treatment and the degree of infiltration of neutrophils and monocytes.

CONCLUSION: sTREM-1 secreted by the gastric mucosa is an independent mechanism connected to the pathogenesis of peptic ulcer. sTREM-1 was released at the presence of *H pylori* from the inflamed gastric mucosa in the field of gastric ulcer.

Key words: sTREM-1; Chronic gastritis; Gastric ulcer; Duodenal ulcer

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INTRODUCTION

Many aspects of the mechanisms implicated in the pathogenesis of peptic ulcer disease remain unclear[1]. Research has been focused more on derangements of mechanisms of protection and repair of the mucosa of the stomach and duodenum[2]. It appears that most cases of both gastric and duodenal ulcer occur in the setting of infection by *H pylori*. Evidence is mounting in support of *H pylori* as a necessary ingredient in the ulcerative process, similar to acid and pepsin. It is not known whether the bacteria or the accompanying inflammation is the most important factor in the pathophysiology of peptic ulcer disease[3].

Triggering receptor expressed on myeloid cells (TREM)-1 is a recently discovered receptor expressed on the surface of neutrophils and monocytes. Engagement of TREM-1 has been reported to stimulate the synthesis of proinflammatory cytokines[4]. A soluble form of TREM-1, named sTREM-1, was observed and identified at significant levels in serum samples of patients with diseases affecting the gastrointestinal tract[5].

sTREM-1 has been found elevated in the gastric juice

of patients with peptic ulcer. Since its levels were positively correlated to the degree of infiltration of the gastric mucosa by neutrophils, leading thus to the hypothesis that sTREM-1 might be a sign of an inflammatory reaction taking place in the gastric mucosa[9]. The latter hypothesis might be strengthened by the lack of expression of TREM-1 on cell membranes of macrophages of healthy intestinal lamina propria[13].

Based on the latter evidence, it is questioned whether sTREM-1 might be produced from the gastric mucosa in the event of peptic ulcer disease. The current study was designed to investigate a role of gastric mucosa for the release of sTREM-1. Furthermore it was investigated whether anti-ulcerative treatment was accompanied by any change of the ability of the mucosa for the secretion of sTREM-1.

MATERIALS AND METHODS

Study group

The study was approved by the Medical and Ethics Committee (6th/11.30.05/26967) of General Hospital "Sismanoglion", Athens, Greece. A total of 72 patients, 54 men and 18 women, aged 58.92 ± 16.52 (mean ± SD) years were enrolled: 56 patients with peptic ulcer disease and 16 patients with chronic gastritis without peptic ulcer. Admitted patients were divided into three groups based on endoscopic findings, as follows: group A: consisting of 35 patients with duodenal ulcer; group B: consisting of 21 patients with gastric ulcer; and group C, consisting of 16 patients with chronic gastritis.

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 done 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 previously described[9]. 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 at the histopathologic findings of each biopsy and after a gastric biopsy culture at the proper growth medium[10]. Exclusion criteria for the study were: recent upper GI bleeding, gastric carcinoma, diabetes mellitus, liver cirrhosis, acute or chronic renal failure and the ingestion of any antimicrobial or antisecretory medication for at least 15 d prior to endoscopy.

Interventions and study design

All patients were examined by upper GI endoscopy. Among patients with duodenal and gastric ulcer disease two endoscopies were performed; the first before treatment and the second 15 d after the end of the treatment. Among patients with chronic gastritis only one endoscopy was done; at each endoscopy biopsies were collected. Gastric juice was aspirated immediately after the entrance of the endoscope into the gastric lumen. At the time of endoscopy, three biopsy specimens were obtained from adjacent areas of the gastric antrum. When each biopsy specimen was taken, the forceps were fully opened 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 *in vitro* culture.

After diagnosis of peptic ulcer disease or gastritis, esomeprasole 20 mg twice daily was prescribed. It was administered for four weeks in patients with duodenal ulcers, for eight weeks in patients with gastric ulcers and for four weeks in patients with chronic gastritis. For patients with infection by *H pylori*, clarithromycin 500 mg bid and amoxicillin 1000 mg bid for 10 d were also added. The above treatment was administered according to international guidelines[11,12].

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[13]. Biopsies were positioned on the insert with mucosal surfaces up. The first biopsy tissue was left unstimulated and served as control; the second was stimulated with 10 ng/mL of lipopolysaccharide of *Escherichia coli* O144:H4 (LPS), and the third with 30 μL of gastric juice of each patient. The total volume of the added growth medium was 2.4 mL; when gastric juice was added it represented 1.25% of the total well volume. At the end of the incubation, the plates were centrifuged for ten minutes at 1400 *g*; then supernatants were collected from the wells and stored at -70℃, until assayed for estimation of sTREM-1 and TNFα. Results were correlated with histopathological findings.

Estimation of sTREM-1

Estimation of sTREM-1 was performed by a home-made enzyme immunoabsorbent assay in samples of supernatants. Capture antibody of sTREM-1 (R&D Inc, Minneapolis, USA) was diluted to 4000 ng/mL and distributed in a 96-well plate at a volume of 0.1 mL per well. After overnight incubation at 25℃, wells were thoroughly washed with a 0.05% solution of Tween in PBS (Merck) (pH: 7.2-7.4). Then 0.1mL of standard concentrations of sTREM-1 (15.1-4000 pg/mL, R & D Inc) diluted with Reagent diluent (1% BSA in PBS, pH 7.2-7.4, 0.2 micron filtered) serving as a buffer or of supernatants was added in wells. After incubation for two hours, wells were washed thrice, and 0.1 mL of one 400 ng/mL dilution of sTREM-1 detection antibody (R&D Inc) was added per well. The plate was then incubated for two hours, and attached antibodies were signalled by streptavidin. Concentrations of sTREM-1 to each well were estimated by the optical density detected at 450 nm after addition of one 1:1 solution of H_2O_2: tetramethylbenzidine as a substrate (R&D Inc). sTREM-1 was expressed in pg/g of tissue. The lowest limit of detection for sTREM-1 was 3.91 pg/g of tissue. All determinations were performed in duplicate; the inter-day variation of the assay was 5.23%.

Estimation of TNFα

Tumor necrosis factor alpha (TNFα) was measured in samples of supernatants with an enzyme immunoabsor-

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Table 1 Demographic characteristics of patients enrolled in the study. Updated Sydney scores are given

Parameters	Pre treatment	Post treatment	Chronic gastritis
N of patients		56	16
Age (mean ± SD)		60.15 ± 17.56	57.11 ± 15.81
Male/Female		46/10	8/8
Non smoking/smoking		16/40	6/10
Gastric ulcer	21	0	-
Duodenal ulcer	35	0	-
History of NSAID use		33/32	4/16
H pylori positive/negative	41/15[a]	7/45[a]	11/5
Patients with evidence of gastritis (Total updated Sydney Score > 0)	49/52[a]	51/52[a]	13/16
Site of gastric inflammation			
Antrum (no of patients)	28	21	8
Corpus (no of patients)	8	6	5
Disseminated (no of patients)	13	7	3
Total updated Sydney score (mean ± SD)	4.69 ± 1.85[a]	2.69 ± 1.22[a]	4.27 ± 0.65
Neutrophil infiltration score (mean ± SD)	1.77 ± 0.39[a]	0.81 ± 0.32[a]	1.73 ± 0.73
Monocyte infiltration score (mean ± SD)	2.13 ± 0.68[a]	1.05 ± 0.61[a]	1.73 ± 0.38
Lymphocyte infiltration score (mean ± SD)	0.87 ± 0.39[a]	0.13 ± 0.09[a]	0.69 ± 0.32
Mucosal atrophy score (mean ± SD)	0.55 ± 0.17[a]	0.38 ± 0.11[a]	0.58 ± 0.30
Intestinal metaplasia (mean ± SD)	0.29 ± 0.04[a]	0.13 ± 0.08[a]	0.19 ± 0.07
Density of *H pylori* (mean ± SD)	1.56 ± 0.28[a]	0.33 ± 0.07[a]	1.45 ± 0.39

[a]*P* vs chronic gastritis, non significant; [b,c]*P* < 0.01, [b]*P* < 0.05, vs pre-treatment scores. [a]*P* vs pre-treatment scores, non significant.

bent assay (EIA, Amersham, London, UK). Lowest limits of detection were 6.25 pg/g of tissue. All measurements were performed in duplicate and cytokine concentrations were expressed as pg/g of tissue.

Histopathology

Formalin-fixed, paraffin-embedded tissue samples were routinely cut at 3-4 μm and stained with haematoxylin and eosin alcian blue PAS (Periodic Acid Schiff) (at pH: 2.5) and Giemsa. Immunochemistry was performed for *H pylori* detection with 1:100 dilution (Biocare Med., California, USA).

The presence of gastritis was evaluated in each biopsy sample after separate scoring for each of the following parameters: (a) disease activity as mucosal infiltration by neutrophils; (b) chronic inflammation expressed as infiltration by monocytes and lymphocytes; (c) degree of mucosal atrophy; and (d) 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 total Sydney score of gastritis ranged between 0 and 15, according to previously reported criteria of the updated Sydney System[15]. The extent of gastric inflammation in the antrum, corpus or both was also recorded. The density of *H pylori* was also evaluated semiquantitatively by the same criteria[15]. Specimens were classified by one pathologist who was unaware of the corresponding laboratory findings.

Statistical analysis

Patients of three groups were further divided into subgroups according to the absence or presence of *H pylori*. Concentrations of sTREM-1, and TNFα were expressed by their median ± 95% confidence intervals (CI) or range. Updated Sydney classification scores were given by their means (± Standard Deviation, SD). 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. Correlations between sTREM-1, and TNFα and the gastritis score or the density of *H pylori* were performed according to Spearman's rank of order. Any *P* value less than 0.05 was considered as significant.

RESULTS

Patients' characteristics are given in Table 1. All patients suffering from duodenal ulcers had presence of *H pylori* on tissue biopsy.

No differences were recorded between patients with duodenal and gastric ulcer disease before treatment and patients with chronic gastritis regarding histological parameters of gastritis according to updated Sydney score. Differences in total updated Sydney score, and several parameters of chronic gastritis before and after treatment among patients with peptic ulcer disease and patients with chronic gastritis are shown in Table 1.

Concentrations of sTREM-1 and of TNFα in supernatants of samples of gastric mucosa taken from patients with either duodenal ulcer disease or gastric ulcer disease or chronic gastritis pre-treatment are shown in Table 2. sTREM-1 was higher in supernatants of tissue samples of *H pylori*-positive than of *H pylori-negative* patients with gastric ulcer. That was also found when mucosa samples were stimulated by LPS. Respectively, similar differences were not found for TNFα. They were also not found for both sTREM-1 and TNFα between *H pylori*-positive and *H pylori-negative* patients with chronic gastritis.

In the above subgroups of patients, concentrations of sTREM-1 were higher in supernatants of gastric mucosa of *H pylori*-positive patients with gastric ulcers than of mucosa of *H pylori*-positive patients with duodenal ulcer after stimulation by LPS ($P < 0.05$). Concentrations of sTREM-1 were also higher in supernatants of gastric mucosa of patients with duodenal ulcer than of patients with gastritis either without or with stimulation by LPS (*P* of comparisons: < 0.01 and < 0.01, respectively). Similar differences for sTREM-1 were found between gastric ulcer and chronic gastritis for *H pylori*-positive patients (*P* of comparisons < 0.01 and < 0.01, respectively). No changes were found when gastric juice was added in cultures. Respective differences in concentrations of TNFα were not observed.

Comparisons of concentrations of sTREM-1 and TNFα between supernatants in unstimulated, and LPS-stimulated samples of gastric mucosa in patients with duodenal and gastric ulcer pre and post treatment respectively, are shown in Table 3. In the presence of LPS, TNFα was increased in supernatants of biopsies taken from *H pylori*

Table 2 Concentrations of soluble triggering receptor expressed on myeloid cells-s (sTREM-1) and tumor necrosis factor-alpha (TNFα) of supernatants of tissue samples taken from patients with either duodenal or gastric ulcer disease or chronic gastritis at pre-treatment

Parameters	Duodenal ulcer		Gastric ulcer		Chronic Gastritis	
	H pylori (+)	*H pylori* (-)	*H pylori* (+)	*H pylori* (-)	*H pylori* (+)	*H pylori* (-)
N	10	0	14	7	9	7
			sTREM-1 [median (range), pg/g of tissue]			
0	32.41 (15.32)	-	317.27 (75.98)[a]	112.41 (36.71)	6.21 (0.01)[c]	5.18 (0.77)
LPS	102.41 (40.43)	-	456.41 (98.20)[a]	214.50 (44.81)	12.87 (2.16)[c]	9.37 (1.50)
Gastric juice	17.91 (7.11)	-	150.14 (41.16)[c]	118.48 (54.90)	10.07 (2.02)[c]	7.42 (1.22)
			TNFα [median (range), pg/g of tissue]			
0	10.62 (2.07)	-	7.56 (1.75)[c]	6.40 (1.52)	8.11 (1.31)[c]	5.98 (1.45)
LPS	28.35 (5.55)	-	45.35 (13.06)[c]	21.44 (8.12)	19.07 (4.72)[c]	17.87 (2.11)
Gastric juice	18.02 (5.97)	-	10.76 (2.06)[c]	9.51 (2.05)	10.17 (3.23)[c]	9.13 (1.61)

Patients were divided as either *H pylori*-positive or *H pylori*-negative. LPS: Endotoxin of Escherichia coli O144:H4. [a]$P < 0.05$, [b]$P < 0.01$, *vs H pylori* negative patients. [c]*P vs H pylori* negative patients. ns: non-significant.

Table 3 Influence of treatment on the secretion of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) and tumor necrosis factor-alpha (TNFα) by the mucosa of *H pylori*-positive patients with gastric and duodenal ulcers

Parameters	*H pylori*-positive with gastric ulcer		*H pylori*-positive with duodenal ulcer	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
		sTREM-1 [median (range), pg/g of tissue]		
0	203.21 (88.91)	8.23 (5.79)[a]	86.82 (35.43)	5.90 (2.84)[a]
LPS	416.87 (127.56)	14.51 (7.11)[a]	144.60 (58.79)	6.15 (4.07)[a]
		TNFα [median (range), pg/g of tissue]		
0	12.67 (226.79)	9.31 (8.02)[a]	32.47 (133.48)	10.31 (20.43)[a]
LPS	31.07 (231.89)	20.12 (13.51)[a]	74.05 (130.56)	17.88 (23.11)[a]

[a]$P < 0.001$, [b]$P < 0.01$, *vs* pre treatment. [c]*P*: non-significant, *vs* pre-treatment.

Table 4 Correlations between sTREM-1 and TNFα and parameters of gastritis score in non and LPS stimulated supernatants of *H pylori* positive patients with duodenal and gastric ulcer and of patients with chronic gastritis

Parameters of Gastritis score	Supernatants of mucosa sampled from *H pylori*-positive patients with duodenal ulcer disease pre-treatment			
	Absence of LPS		Presence of LPS	
	sTREM-1	TNFα	sTREM-1	TNFα
Neutrophils infiltration	P = NS	P = NS	P = NS	P = NS
Monocytes infiltration	P = NS	$P < 0.01$	$P < 0.05$	$P < 0.01$
	Supernatants of mucosa sampled from *H pylori*-positive patients with gastric ulcer disease pre-treatment			
Neutrophils infiltration	P = NS	$P < 0.05$	$P < 0.01$	$P < 0.05$
Monocytes infiltration	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
	Supernatants of mucosa sampled from *H pylori*-positive patients with chronic gastritis			
Neutrophils infiltration	P = NS	$P < 0.05$	P = NS	$P < 0.05$
Monocytes infiltration	P = NS	$P < 0.05$	P = NS	$P < 0.01$

negative patients with gastric ulcer before treatment and with chronic gastritis ($P < 0.05$ and < 0.05, respectively).

Correlations of sTREM-1 and TNFα between supernatants of patients with duodenal and gastric ulcer pre treatment and of patients with chronic gastritis when cultured in the absence/presence of LPS and parameters of gastritis score are shown in Table 4. In the absence of LPS sTREM-1 concentrations were significantly correlated with monocytes infiltration in *H pylori* positive patients with gastric ulcer. In the presence of LPS sTREM-1 was significantly correlated with both monocytes and neutrophils infiltration in *H pylori* positive patients with gastric ulcer, respectively, significant correlations were also observed between sTREM-1 and monocytes infiltration in *H pylori* positive patients with duodenal ulcer. Respectively, both in the absence/presence of LPS TNFα concentrations were significantly correlated with monocytes and neutrophils infiltration in *H pylori* positive patients with and gastric ulcer, respectively, significant correlations were also observed between TNFα and monocytes infiltration in *H pylori* positive patients with duodenal ulcer. No significant correlation was found between any of the latter scores and sTREM-1 or TNFα of supernatants of biopsies taken from patients post-treatment.

DISCUSSION

Among patients suffering from chronic active gastritis only a minority evolves to peptic ulcer disease[19], rendering the question what might be the underlying mechanism leading several patients with gastritis to peptic ulcer and others not. Recent data revealed that sTREM-1 was found increased in the gastric juice of patients with peptic ulcer disease compared to patients with chronic gastritis[13]. That finding might lead to the hypothesis that sTREM-1 might constitute an independent factor leading from gastritis to peptic ulcer.

The present study applied a unique design. It is the first time, to our knowledge, in the literature where the ability of

4614 ISSN 1007-9327 CN 14-1219/R World J Gastroenterol September 14, 2007 Volume 13 Number 34

the gastric mucosa for the release of sTREM-1 and TNFα was studied among patients with either duodenal ulcer or gastric ulcer or chronic gastritis without signs of peptic ulcer disease. Supernatants of biopsies taken from the enrolled patients were stimulated with LPS and gastric juice of patients. It is known that *H pylori*-as a Gram negative bacterium secretes LPS that mediates to the gastric inflammation[16]. As described by others, cell loss and apoptosis of gastric mucous cells was enhanced by *H pylori* LPS with less potency compared to the same effect by *E. coli* LPS. The low immunological potency of *H pylori* LPS may contribute to low grade gastritis[17]. In an attempt to simulate the latter process cultured biopsies were stimulated with LPS. Inflammation of the gastric mucosa was significantly reduced after treatment whereas *H pylori* was also eradicated (Table 1). Although the proper treatment was administered in patients with chronic gastritis second endoscopy was not performed; the latter was thought to be of no significance because sTREM-1 concentrations were already minimal pre treatment.

Results revealed that gastric mucosa of *H pylori* positive patients with both duodenal and gastric ulcer disease was potent to secrete sTREM-1. The potency for secretion of sTREM-1 was lost post-treatment. The release of sTREM-1 was higher by *H pylori* infected gastric mucosa than by gastric mucosa not infected by *H pylori*. The effect of *H pylori* on the release of sTREM-1 by mucosa of patients with duodenal ulcer could not be assessed since all patients with duodenal ulcer in the studied cohort were *H pylori*-positive.

Similar kinetics to sTREM-1 were not found for TNFα. TNFα was found increased in strict correlation with the degree of mucosal inflammation independently from the underlying pathogenetic status. The latter was highlighted by the fact that TNFα was increased post treatment when gastric mucosa was stimulated by LPS (Table 4).

The main assumption revealed from the presented study was that sTREM-1 was secreted by the activated inflammatory cells that infiltrate the gastric mucosa; inflammatory cells were immigrated to the inflamed gastric mucosa attracted by *H pylori* or its components. The treatment of inflamed gastric mucosa and the eradication of *H pylori* ceased the secretion of sTREM-1. It is of great importance that the latter assumption exists only in the status of gastric and duodenal ulcer disease. The release of sTREM-1 was independent from the density of mucosal inflammation at patients with no evidence of peptic ulcer. The pattern of release of sTREM-1 by the activated inflammatory cells and probably their intracellular activity should be further investigated.

The independent contribution of sTREM-1 release in the pathogenesis of gastric ulcer is further aggravated by the observation that gastric juice could not influence the activity of the inflamed mucosa per se. Stimulation of inflamed gastric mucosa with gastric juice was not lead to a significant increase to the release of sTREM-1 and TNFα (Table 4).

The present study revealed for the first time in the literature that sTREM-1 secreted by the gastric mucosa is an independent mechanism connected to the pathogenesis of gastric and duodenal ulcer. sTREM-1 was released at the presence of *H pylori* from the inflamed gastric mucosa in the field of gastric ulcerative process. The exact pathogenetic mechanisms needs to be further clarified.

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Original Paper

Evaluation of a Conventional ELISA (Novitec®) and a Near Patient Immunochromatographic Test (Stick H. pyl®) for Helicobacter pylori Antigen Detection in Stool

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ABSTRACT

Background/Aims: The detection of *Helicobacter pylori* (*H. pylori*) antigen in stool by conventional ELISA is a reliable non-invasive method for the diagnosis of *H. pylori* infection in untreated patients. Recently, rapid in-office stool tests have been developed for the same purpose.

Methodology: We have prospectively evaluated the performances of a commercially available enzyme-linked immunoassay (Novitec® EIA) and a rapid near-patient immunochromatographic stool test (Stick H Pyl®) for the detection of *H. pylori* stool antigen. Fifty *H. pylori* positive and 50 negative patients were included. *H. pylori* infection was diagnosed by using histology, rapid urease test and urea breath test. Patients were classified as positive if two of the three tests were positive and negative if all the three tests were negative. Testing was carried out according to the manufacturers' instructions.

Results: Novitec® EIA had 8% equivocal results. If they were interpreted as negative the sensitivity, specificity, positive predictive value, negative predictive value and overall diagnostic accuracy were 82%, 86%, 86%, 83% and 84% and if as positive 88%, 76%, 79%, 86% and 82% respectively. ROC curve analysis showed a cut-off value of 0.144 for our population. The corresponding numbers for this cut-off value were: 82%, 94%, 93%, 84% and 88%. The respective numbers for Stick H Pyl® were 78%, 78%, 76%, 78% and 79%.

Conclusions: Novitec® EIA performed well in this cohort of Greek patients and demonstrated a high specificity and positive predictive value when we adjusted the cut-off at 0.144. Performance of Stick H Pyl® was sub-optimal.

KEY WORDS: *Helicobacter pylori*; Antigen stool test; Enzyme immunoassay

ABBREVIATIONS: *Helicobacter pylori* (*H. pylori*); Urea Breath Test (UBT); Positive Predictive Value (PPV); Negative Predictive Value (NPV); Overall diagnostic accuracy (ODA); Receiver Operator Characteristics (ROC); Optical Density (OD); Delta Over Base (DOB);

INTRODUCTION

Helicobacter pylori (*H. pylori*) is the major cause of gastritis, peptic ulcer disease and is also associated with gastric cancer and gastric MALT lymphoma (1-3). Although its prevalence continues to fall in countries with high socio-economic level, it remains a major health problem worldwide (4). Several methods both invasive and non-invasive are used in the clinical practice for the diagnosis of *H. pylori* infection (5). Rapid urease test and histology of the gastric mucosa are the most commonly used invasive methods (5). They are highly accurate in diagnosing the infection but their major drawback is that they require endoscopy. Non-invasive methods include urea breath test (UBT), serology and stool antigen test and are the preferred methods in young untreated dyspeptic individuals without alarm symptoms (6). UBT is very accurate but it is expensive and requires specialized equipment which is not always available in the primary care setting (7). Enzyme-linked immunoassays for the detection of *H. pylori* stool antigen are sensitive and specific in diagnosing the infection and unlike serology they can provide evidence for present rather than past infection (6). Recently near-patient rapid immunochromatographic stool tests have been suggested as a method for providing rapid in-office diagnosis for *H. pylori* infection (8).

In the present study we aimed to validate the performance of a commercially available stool enzyme-linked immunoassay (Novitec® EIA, Germany) and a rapid immunochromatographic test (Stick H. pyl®, Zaragoza, Spain) in untreated Greek patients. Both kits are manufactured in Europe. According to the manufacturers, both methods are highly sensitive and specific (>90%) but a formal evaluation of their performance has not been published so far.

Hepato-Gastroenterology 2007; 54:797-800

METHODOLOGY

We prospectively studied outpatients 50 positive and 50 negative for *H. pylori* who underwent gastroscopy for dyspeptic symptoms. Exclusion criteria were the following: age <18 years, previous treatment for *H. pylori* eradication, use of proton pump inhibitors, bismuth compounds or antimicrobials for the last 4 weeks prior to inclusion, previous gastrectomy, gastric cancer, pregnancy and lactation and patient's refusal for participation.

Patients were instructed to bring a fresh stool sample in the morning of the endoscopy. Stool samples were stored at -20°C until testing. During endoscopy three biopsy specimens were taken-one from the lesser curve, two cm from the pylorus for the rapid urease test (CLO test) and two (one from the antrum and one from the body) for histology and modified Giemsa stain. The result of the rapid urease test was read at two hours by a single trained observer who was blinded to the endoscopic findings and the results of the UBT. A single experienced histopathologist assessed the presence of *H. pylori*. All patients underwent urea breath test (UBT) with 75mg of urea ^{13}C (Helicobacter test INFAI, Bochum, Germany). A baseline breath sample was taken and then urea was administered with orange juice. A second breath sample was taken 30 minutes after urea ingestion. The $^{13}C/^{12}C$ ratio (Delta) was automatically calculated by using infrared spectrometry (FANci2, Olympus, Europe). A Delta over Base (DOB) value of >4‰ was considered positive.

The study was approved by the local ethics committee and patients signed informed consent prior to inclusion.

Gold Standard

Patients were considered *H. pylori* positive if they tested positive for at least two of the three tests (CLO test, histology, UBT) and negative if all the tests were negative. Equivocal cases were excluded.

Detection of *H. pylori* Stool Antigen by the Novitec® EIA

The assay was performed according to the manufacturer's instructions. Stool samples were thawed and testing was performed at room temperature. A stool sample (5-6mm in diameter) was mixed with 400μL of sample diluent in test tubes and the preparation was emulsified by vortexing vigorously for 20 seconds. Watery stools were considered inappropriate for testing. A small quantity (100μL) of each diluted stool sample was transferred to the bottom of antibody-coated microwells and 100μL of conjugate (specific rabbit polyclonal antibody conjugated to horseradish peroxidase) were added. In 2 wells 100μL of positive and negative control respectively were added. The mixture was incubated at room temperature for 1 hour. After incubation the liquid content was dumped out and the wells were washed 6 times with 200mM phosphate buffer solution. Substrate (3,3', 5,5' tetramethylbenzidine) was subsequently added. The reaction was stopped at 10 minutes by using 1N sulphuric acid and spectrophotometric determination followed at 450/650 nm wavelength. According to the manufacturer, an optical density (OD) >0.121 is positive, <0.100 negative whilst any interim value is equivocal.

Stick H pyl®

Simple H. pyl® was performed by an investigator blinded to the *H. pylori* status as well as to the results of Novitec® EIA and UBT. A 5- to 6-mm stool sample was added to the vials containing 1mL of diluent and emulsified by vortexing for 15 seconds. The tip of each vial was snapped and 4 drops were added to the sample port of the test cassette. The result was read after 5 minutes incubation at room temperature. Tests were considered negative if there was only a single line in the control window and positive if a second line appeared in the test window.

Statistical Analysis

The sensitivity was calculated as the proportion of positive tests in patients with *H. pylori* infection according to the gold standard and specificity as the proportion of negative tests in patients who tested negative for *H. pylori*. Positive predictive value (PPV) was the proportion of true positive tests among all the positive tests whilst negative predictive value (NPV) the proportion of true negative tests among all the negative tests. Overall diagnostic accuracy (ODA) was calculated as the proportion of true positives plus true negatives among all tests. Chi-square test with Yates' correction was used to compare the performance characteristics of the 2 tests. The receiver operator characteristics (ROC curve) and the optimal cut-off for Novitec® EIA were also calculated. Statistical analysis was performed by using the statistical package SPSS 8.0 for Windows.

RESULTS

The mean age of the patients was 55.8 years (range 23-80). There were 53 men and 47 women. Twenty-three patients had duodenal ulcer, 8 had gastric ulcer, and the 69 non-ulcer dyspepsia.

Novitec® EIA

There were 8 equivocal results generated by Novitec® EIA **(Table 1)**. If equivocal results were considered negative, the sensitivity, specificity, PPV, NPV and ODA for Novitec® EIA were 82%, 86%, 86%, 83% and 84% respectively **(Table 2)**. In the opposite case, the respective numbers were 88%, 76%, 79%, 86% and 82%. ROC curve analysis showed a cut-off value of 0.144 (area under the curve 0.907) **(Figure 1)**. The performance characteristics of Novitec® EIA according to the cut-off found by ROC curve analysis were 82%, 94%, 93%, 84% and 88% respectively **(Table 2)**.

Stick H. pyl®

The sensitivity, specificity, PPV, NPV and ODA were 78%, 78%, 76%, 78% and 79% respectively **(Table 2)**. Although the procedure was easy to per-

TABLE 1 Comparative Results of Novitec® EIA and Stick H. pyl®

		Result	Patient status (n)
		Positive (50)	Negative (50)
Novitec®	Positive	41	7
	Negative	6	38
	Equivocal	3	5
Simple H. pyl®	Positive	39	11
	Negative	11	39

form the main disadvantage of the test was the weakly positive bands which were difficult to interpret. From the 50 positive bands 14 were weakly positive (10 in patients with positive and 4 in patients with negative *H. pylori* status). All these bands were developed between 5 and 10 minutes incubation time. The proportion of weakly positive bands in the true positive results was 25.6%. The OD generated by Novitec® EIA in the cases with weakly positive bands showed 2 equivocal, 7 positive and 5 negative results.

Cost of the Non-invasive Procedures

The cost per patient was 55.7 Euros for the UBT, 24.2 for Novitec® EIA and 23.8 for Stick H. Pyl®.

DISCUSSION

H. pylori has a significant impact on clinical practice. According to the Maastricht (Europe) and the National Institute of Health (USA) consensus recommendations, the "test and treat" strategy should be preferred in patients <45 years without alarm symptoms (9). Avoiding endoscopy in this particular group of patients seems to be a cost-effective practice as it reduces endoscopy workload, allowing the management of such individuals by the primary care physicians. Non-invasive methods for the diagnosis of *H. pylori* infection are comparable to invasive tests in terms of sensitivity and specificity but most of them require laboratory facilities. Cost effectiveness studies have shown that when the prevalence of *H. pylori* is intermediate or low (<60%), ELISA serology is inaccurate compared to stool tests and UBT (10). The latter option continues to be expensive in Greece compared to other non-invasive methods and the necessary equipment does not always exist in the primary care setting. The use of in-office stool tests seems to be an attractive approach as they do not require specialized equipment and the result can be read within several minutes.

We validated two commercially available stool tests in the Greek population, i.e. an enzyme-linked immunoassay and a rapid immunochromatographic test. The performance of Novitec® EIA was optimal and despite the use of polyclonal antibody specificity was significantly higher compared to Simple H. Pyl®. Unpublished data (11) of the performance characteristics of Novitec® EIA show a sensitivity of 96% which is considerably higher than the sensitivity found in our study. This discrepancy and the relatively high rate of equivocal results (8% of the tests) may be explained by the different populations under investigation as it has been suggested that *H. pylori* antigen can be diluted by the large stool volume of patients from certain geographical regions who consume more fiber in their diets (12).

Five out of eight of the equivocal results of Novitec® corresponded to *H. pylori* negative patients. The interpretation of these equivocal results as negative was associated with lower sensitivity but a higher specificity. However, if equivocal results were considered positive, specificity was unacceptably low (76%). When results were adjusted according to the cut-off found by ROC curve analysis, sensitivity was 82%, specificity 94% and ODA 88% which agrees with published studies and shows that the cut-off of every enzyme-linked immunoassay on *H. pylori* should be adjusted according to the population under study (13). The high PPV of Novitec® EIA as a consequence of the high specificity reduces the proportion of false positive tests and therefore a young dyspeptic patient testing positive can be safely presumed to be positive and thus endoscopy can be avoided.

The performance of Stick H. Pyl was not optimal. According to the manufacturer, the test is based on a monoclonal antibody which binds a specific *H. pylori* antigen. Although assays based on monoclonal antibodies tend to be less sensitive but more specific (5), this did not seem to be the case for Simple H. Pyl® as its specificity was only 78%. A similar study comparing

TABLE 2 Performance Characteristics of Stick H. Pyl® and Novitec EIA according to the Cut-off Used

Novitec® EIA	Cut-off value	Sensitivity	Specificity	PPV	NPV	ODA
	0.121[1]	82%	86%	86%	83%	84%
	0.121[2]	88%	76%	79%	86%	82%
	0.144	82%	94%*	93%*	84%	88%
Stick H. Pyl®		78%	78%*	76%*	79%	

[1]Equivocal results were considered negative; [2]Equivocal results were considered positive; *$p = 0.002$. The asterisks indicate statistically significant results.

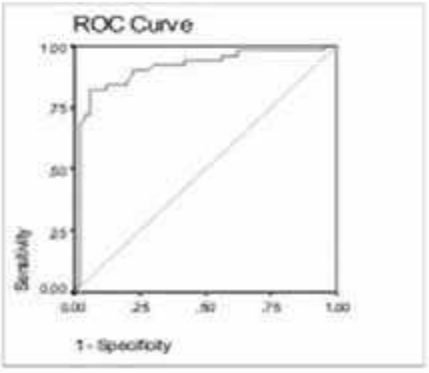

FIGURE 1
ROC curve of Novitec EIA plotted for different cut-off points. Each point represents a sensitivity/specificity pair corresponding to a particular decision threshold.

this rapid immunochromatographic test with the HpSA EIA (Meridian Diagnostics Ohio), found excellent sensitivity and better reliability than HpSA but again specificity was 60-70% (14).

Two studies (5,15) evaluated another rapid immunochromatographic stool test (ImmunoCard STAT HpSA) and found higher overall accuracy than that of Simple H. Pyl in our study. However, similarly to our study, there was a difficulty in interpreting weakly positive bands (5). This problem may well be the case for most immunochromatographic tests as the result is visual and can be interpreted differently by individuals performing them especially if they are general practitioners with poor laboratory experience. All the weakly positive bands in our study required 5-10 minutes to appear, which is longer than the incubation time recommended by the manufacturer.

In conclusion, Novitec EIA® showed reasonable sensitivity, high specificity and PPV and can be used reliably in the context of the "test and treat" strategy in young dyspeptic individuals. We suggest the cut-off of 0.144 as optimal for the Greek population. The rapid immunochromatographic stool test Simple H. Pyl® did not perform well in our study and therefore improvement of the test's characteristics is needed.

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Perspectives

Parental Family Structure, *Helicobacter Pylori*, and Gastric Adenocarcinoma

Pagona Lagiou*, Dimitrios Trichopoulos

The study of family of origin (parental family), as contrasted to family of procreation, originally received attention from epidemiology in the context of childhood infectious diseases. Two aspects of parental family have been intensely investigated: sibship size and birth order [1,2]. Larger sibship size increases the likelihood of introduction and spread of infectious agents within the family and tends to be inversely associated with children's average age at infection. Birth order, in the absence of vaccination, has a more specific effect on average age at infection, since first-born children are usually infected when first exposed to the child care or school environment, whereas later-born children tend to be infected earlier, even in utero, by their older siblings.

Parental Family and *Helicobacter Pylori*

Larger sibship size and higher birth order have long been known to be associated with higher prevalence of chronic infections by the hepatitis B virus [3]. More recently, similar associations have been reported for infection with *Helicobacter pylori* [4]. Clearly, since these microorganisms are etiologically related to hepatocellular carcinoma and gastric adenocarcinoma, respectively, sibship size and birth order should be—and have been reported to be [5,6]—risk factors for these malignancies.

A more subtle question, however, is the following: given chronic carrier state, does larger sibship size and higher birth order, as indicators of earlier infection, predict higher risk of cancer at the corresponding site? For hepatitis B virus and hepatocellular carcinoma this question has been affirmatively answered [7], and the new study by Blaser and colleagues published in *PLoS Medicine* goes a long way in documenting a similar association for *H. pylori* and gastric adenocarcinoma [8].

The New Study

In a cohort of 9,935 Japanese-American men, who had provided blood samples at entry in the study and were followed for 28 years, 261 cases of non-cardia gastric cancer were identified. In a nested case-control design, each case patient was paired with one control, matched for age at examination and date of serum collection. Of the 261 patients with non-cardia gastric cancer, 239 (92%) were carriers of *H. pylori* on the basis of antibody status and 189 (72%) carried *cagA*+ strains, which are considered more virulent. The corresponding numbers and proportions among the 261 matched controls were 205 (79%) and 155 (59%). In line with the study objective, further analyses were restricted to patients who were serologically positive for *H. pylori* in general or *cagA*+ in particular cases and controls.

Earlier age at establishment of *H. pylori* carrier state increases the risk of gastric cancer decades later.

Gastric cancer cases were further distinguished, on the basis of Lauren's classification [9], into the more common intestinal type, the less common diffuse type, and the uncommon mixed, other, and unknown types. Among those who were *H. pylori* positive, there was a significant positive association of gastric adenocarcinoma with sibship size, which was somewhat more evident for the diffuse type. With respect to birth order, a borderline significant positive association was only evident for the intestinal type. Results for both sibship size and birth order were generally more striking when analyses were limited to *cagA*+ study participants.

The results of the study by Blaser and colleagues [8] strongly indicate that earlier age at establishment of *H. pylori* carrier state increases the risk of gastric cancer several decades later. There are several plausible explanations considered by the authors, but the empirical evidence implicating early intra-familial transmission points to intra-familial selection of better adapted and more virulent strains [10] in a background of age-modulated immune response.

Strengths and Limitations of the Study

Blaser and colleagues [8] have used an important cohort to evaluate evolving concepts on the natural history of *H. pylori* infection and have reached conclusions that are of both theoretical and practical importance. A limitation of their study is the relatively small number of cases with gastric adenocarcinoma but, clearly, not much could be done about this. Another limitation is that mutual

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control of sibship size and birth order was not attempted. These two variables are strongly positively correlated (one cannot have a high birth order unless one belongs in a large sibship) and association of gastric cancer with just one of them would be reflected in an association with the other as well. In this instance, however, since increases in both of these variables tend to reduce age at infection, the reported associations point to a biologically sound and valuable conclusion.

Whether sibship size or birth order is the driving force in the association of early age at establishment of chronic *H. pylori* infection with gastric adenocarcinoma, socioeconomic status plays a critical role. This is because in most settings low socioeconomic status is associated with larger sibship size and modulates the consequences of birth order by intensifying the intra-familial transmission of contagious agents. The results of the investigation by Blaser and colleagues, however, allow a better insight into past trends of gastric cancer incidence and may improve predictions of future trends by introducing into the equation two additional, albeit interrelated, explanatory variables.

Next Steps

A broader lesson from this study is that age at infection or establishment of chronic carrier state is a variable of considerable importance in assessing the carcinogenic potential of infectious agents. These consequences are unlikely to be uniform across the various cancer types—we already have evidence that late infection with unspecified agent(s) increases the risk of childhood leukemia [11,12]. Identifying the agents' molecular characteristics or the hosts' immunological aspects in early or late infection is a field that merits further attention in the study of cancer etiology. ■

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Pathogenesis of *Helicobacter pylori* Infection

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Abstract

The clinical outcome of *Helicobacter pylori* infection is determined by a complex interaction between the bacterium and the host. The main bacterial factors associated with pathogenicity comprise outer membrane proteins, including BabA, SabA, OipA, AlpA, and AlpB, the vacuolating cytotoxin VacA and the products of *cag*PAI. The multitude of putative virulence factors makes it extremely difficult to test the contribution of each individual factor. Much effort has been put into identifying the mechanism associated with *H. pylori*-associated carcinogenesis. Interaction between bacterial factors such as CagA and host signal transduction pathways seems to be critical for mediating cell transformation, cell proliferation, invasion, apoptosis/anti-apoptosis, and angiogenesis. An animal model using the Mongolian gerbil is a useful model for showing gastric pathology due to *H. pylori* infection which is similar to that in humans and can be used to evaluate virulence factors including CagA, host responses, and environmental factors such as salt intake.

Colonization and Adherence

The majority of *Helicobacter pylori* cells are found in the gastric mucus layer overlying the epithelium; however, the organism has been reported to interact with gastric epithelial cells and even invade them. *H. pylori* biofilm formation has recently been reported to be involved in the colonization of the human stomach. In biopsies from human gastric mucosa, mature biofilms covering almost the entire mucosal surface were observed in urease-positive patients, while coverage was less than 2% in urease-negative patients [1]. *H. pylori* adherence to gastric epithelial cells facilitating access to nutrients and delivery of effector molecules has been considered essential for development of the disease. Several of the *H. pylori* Hop proteins (outer membrane proteins) have been identified as adherence factors, including BabA, SabA, OipA, AlpA, and AlpB; however, their exact role in *H. pylori* pathogenicity remains unclear. Unlike previous reports, *H. pylori* strains expressing low levels of BabA contributed to more severe mucosal injury and were more frequently associated with duodenal ulcer (DU) and gastric cancer (GC) than strains with a high-level expression of BabA or those lacking the *bab*A gene. Moreover, Le(b)-binding activity or the presence of *H. pylori* strains with triple-positive status (*cag*A+/*vac*As1/*bab*A-H) did not accurately reflect the severity of mucosal damage or relate to the clinical outcome [2]. Colbeck et al. found extensive genotypic diversity in *bab*A and *bab*B among different strains, as well as in a strain colonizing an individual patient, which may reflect selective pressures for adherence [3].

Dossumbekova et al. [4] reported that *hop*H (*oip*A) mutagenesis resulted in lower adherence to gastric epithelia in vitro, but did not alter the epithelial interleukin (IL)-8 secretion and confirmed previous observations that in-frame ("on" genotype) *hop*H alleles are associated with the presence of *vacAs1*, *vacAm1*, *bab2*, and *cag*A genotypes. OipA-positive expression status was significantly associated with the presence of DUs and GC, high *H. pylori* density, and severe neutrophil infiltration, while SabA positive status was associated with GC, intestinal metaplasia as well as corpus atrophy, and negatively associated with DU and neutrophil infiltration [5]. Another major point was that SabA expression frequently switched "on" or "off", suggesting a response to changing conditions in the stomach. Lu et al. [6] confirmed that AlpAB may be involved in cellular adherence as whole *alpA*/*alpB*-deleted mutants were poor colonizers of the stomachs of C57BL/6 mice, and were associated with lower mucosal levels of proinflammatory effectors KC and IL-6. Interestingly, deletion of *alp*AB reduced IL-8 induction by AGS cells following infection with East Asian but not with Western strains.

 Helicobacter 12 (Suppl. 1): 10–14

*cag*PAI, VacA

H. pylori strains with more EPIYA motifs induce higher levels of CagA phosphorylation and more cytoskeletal rearrangements, which are associated with atrophic gastritis and GC. By sequential immunoprecipitation and immunoblot, it was shown that: (1) CagA proteins carrying multiple EPIYA-C or EPIYA-D sites bound to and deregulated SHP-2 more strongly than those with a single EPIYA-C or EPIYA-D, and that (2) the ability of CagA to bind to Csk was correlated with the number of EPIYA-A and EPIYA-B sites [7]. Ren et al. [8] reported that CagA multimerization within the EPIYA-C segment as well as in sequences located immediately downstream of the EPIYA-C or EPIYA-D segments is a prerequisite for CagA-SHP-2 interaction and subsequent deregulation of SHP-2. However, from a clinical perspective, there was no significant correlation between the number of EPIYA motifs or CagA subtypes and various gastroduodenal diseases [9]. Isogenic *H. pylori* strains, from the same patient, expressing CagA with different numbers of EPIYA-C repeats have been reported to exist in approximately 10% of the population and should be taken into consideration during routine EPIYA screening of *H. pylori* isolates [10].

The *H. pylori vacA* gene encodes a secreted protein (VacA) that has been reported to exhibit a pleiotropic activity on gastric epithelial cells as well as on T lymphocytes. Shirasaka [11] proposed that VacA binding to its receptor PTP zeta/beta results in gastric epithelial detachment and eventually gastric ulceration through abnormal signaling. Based on their data, Terebiznik et al. [12] proposed that VacA-dependent *H. pylori*-containing vacuoles protect the bacterium from the bactericidal components of the lysosomal pathway, promoting bacterial survival and contributing to the persistence of infection.

Other Potential Virulence Factors

Potential new markers associated with disease-related strains include the gene jph0870 [13] and outer membrane proteins Omp26, Omp30, and Omp6 [14], the gene jph0562 for LPS biosynthesis [13], and the hp0015 homolog of *H. pylori* strain 26695 [15]. Lin et al. [16], using two-dimensional immunoblots, identified elongation factor EF-G (FusA), catalase (KatA), and urease alpha subunit (UreA) as DU-related antigens, showing a higher seropositivity in DU samples than in GC. A novel lipolytic enzyme (EstV) encoded by ORF HP0739 of *H. pylori* 26695, isolated, cloned, and purified by Ruiz et al. [17], was proposed to be involved in mucus degradation and the release of proinflammatory and cytotoxic compounds. Finally, Baldwin et al. [18], by monitoring the colonization ability of transposon mutants of two *H. pylori* strains in a mouse model, identified 10 previously uncharacterized colonization gene loci candidates.

H. pylori Infection and Signal Transduction Pathway

Activation of signal transduction pathways caused by *H. pylori* infection has been studied extensively in recent years. Nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) activation are the critical regulators of innate immune responses and inflammation. Pathak et al. found that HP0175, which is a peptidyl prolyl *cis*-, *trans*-isomerase, was capable of interacting directly with the extracellular domain of Toll-like receptor 4 (TLR4). HP0175-induced IL-6 gene expression was critically dependent on TLR4-dependent NF-κB and MAPK activation in monocytic cells [19]. Obonyo et al. also showed that TLR2 or TLR4 is required for *H. pylori*-induced cytokine expression such as IL-1β and IL-6 in macrophages [20]. Moreover, Zhao et al. noted that NF-κB and MAPK signaling linked to the TLR2 might be necessary for *H. pylori*-HSP60-induced IL-8 secretion [21]. These observations suggest that TLR-mediated signal transduction pathways such as NF-κB and MAPK are critical for the pathogenesis of *H. pylori* infection. Interestingly, several reports revealed that polymorphisms in genes related to bacterial lipopolysaccharide/peptidoglycan signaling such as TLR or CD14 may be implicated in the development of gastric premalignant lesions and GC [22–24]. Cho et al. analyzed the crosstalk between MAPK and NF-κB activation by *H. pylori* and found that extracellular signal-regulated kinase (ERK) induced phosphorylation of IκBα in *H. pylori*-infected AGS cells [25].

Dysregulation of apoptotic and anti-apoptotic pathways has been recognized as an important factor in *H. pylori*-mediated pathogenesis [26]. *H. pylori*-infected antrum showed greater surface epithelial apoptosis that decreased after eradication therapy [27]. In vitro, *H. pylori*-induced apoptosis of T cells is mediated by the mitochondrial pathway and might necessitate a local environment that facilitates life-long infection [28]. In addition to the previous reports, virulence factors such as VacA, gamma-glutamyltranspeptidase, external membrane vesicles, have been shown to be inducers for apoptosis [29–31]. An anti-apoptotic function of *H. pylori* has also been reported. Low multiplicity of *H. pylori* infection suppresses apoptosis of B lymphocytes and suggests that the low levels of infection that occur in the human stomach are associated with cell survival, proliferation, and development of mucosa-associated lymphoid tissue lymphoma [32].

CagA/*cag*PAI and Host Response

As previously described, the CagA protein, an *H. pylori* virulence factor, induces morphologic changes in host cells.

and may be associated with the development of peptic ulcer and GC. The mechanism concerning how CagA contributes to the pathogenesis of *H. pylori* infection is still obscure. Murata-Kamiya et al. reported that CagA can interact with E-cadherin independently of CagA tyrosine phosphorylation. This interaction impairs the complex formation between E-cadherin and β-catenin and causes nuclear accumulation of β-catenin. Dysregulated β-catenin transactivates genes such as cdx1, which encodes an intestinal specific CDX1 transcription factor, and may be implicated in the development of intestinal metaplasia [33]. They also reported that FAK, via SHP-2, plays a crucial role in the morphogenetic activity of CagA, and impaired cell adhesion and increased motility may be involved in the development of gastric pathology due to *H. pylori* infection [34]. Poppe et al. identified the nonreceptor tyrosine kinase c-Abl as a crucial mediator of *H. pylori*-induced migration and a novel CagA kinase in epithelial cells. C-Abl interacts directly with CagA and is localized in focal adhesion complexes and membrane ruffles, which are dynamic cytoskeletal structures necessary for cell motility [35].

Chang et al. explored the effect of CagA on the expression of cyclin D1, an important cell cycle regulator. *H. pylori*-induced cyclin D1 expression was attenuated in a *cagA* mutant, and AP1 and cAMP response elements (CRE) were involved in the induced cyclin D1 expression [36].

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine implicated in carcinogenesis. Beswick et al. revealed that *H. pylori* induced MIFs which were dependent on CagA. MIF can bind to CD74, which is highly expressed on the surface of gastric epithelial cells. They also investigated the role of the *H. pylori*-induced MIF on epithelial proliferation and procarcinogenic events and found that recombinant MIF and *H. pylori* increased cell proliferation. Proliferation was decreased when *cagA*-negative strains were used and when CD74 was blocked by mAbs. Furthermore, MIF binding to CD74 was also shown to decrease p53 phosphorylation and up-regulate Bcl2 expression [37].

A relationship between CagA and pro- and anti-apoptosis factors has been reported. Infection with *cagA*-positive strains is associated with an over-expression of pro-apoptotic proteins in the gastric mucosa, mainly at the antral lesser curvature, which may play a role in atrophy development [38]. Zhu et al. investigated whether CagA modulates the activation of MAPKs and their downstream apoptosis regulators in B lymphocytes. Transfection of lymphocytes with *CagA* transiently increased Erk1/2 phosphorylation, which was negatively regulated by MAPK phosphatases, MKP-1 and MKP-6. Activation of MAPK led to phosphorylation of Bad at Ser-112 which plays a role in anti-apoptosis [39].

The role of *cag*PAI in *H. pylori* cellular invasion remains controversial. Oliveira et al. [40] reported, in agreement with previous observations, that the invasion of AGS cells was a bacterial T4SS-dependent event involving the activation of c-Met receptors and the up-regulation of matrix metalloproteinase (MMP)-2 (gelatinase-2) and MMP-9 (gelatinase-9) activity. However, Kundu et al. [41] reported that a strain lacking the *cag*PAI and the strain SS1 with a non-functional CagPAI was equipotent in increasing pro-MMP-9 induction, but affected MMP-2 activity only moderately. They also provided data showing that up-regulation of MMP-9 may be mediated via proinflammatory cytokines through different pathways other than *cag*PAI.

Pathogenesis of *H. pylori* in an Animal Model

The Mongolian gerbil is a useful model because the gastric pathology caused by *H. pylori* infection in gerbils is similar to that in humans. Shibata et al. [42] reported that although the stomachs of Mongolian gerbils were colonized at similar densities by wild-type *H. pylori* strains and isogenic mutants with disrupted *cagA*, or *cagE* genes, the Δ*cagA* mutant induced milder gastritis. Kudo et al. analyzed the role of transcription factors in the gastric mucosa of *H pylori*-infected gerbils and observed that the gastric mucosal transcription factors induced by *H. pylori* infection differed according to the phase and outcome of infection. AP-1 and CREB levels were early detectors related to inflammation and ulceration, whereas NF-κB and ISRE were late detectors related to atrophy [43]. Caco et al. demonstrated that the term and severity of *H. pylori* infection might play important roles in gastric carcinogenesis, with an essential involvement in chronic inflammation [44]. Kato et al. studied dose-dependent enhancing effects of salt in chemical carcinogenesis in *H. pylori*-infected gerbils and found that a reduced salt intake could be one of the most important chemopreventive methods for human gastric carcinogenesis [45]. Mongolian gerbils seem to be a very valuable model for *H. pylori* infection; however, Otaka et al. reported that Mongolian gerbils might be special animals in which HSP70-induction is absent and the mucosal protective ability in HSP-dependent cytoprotection in the gastric mucosa is extremely poor. They suggest that the Mongolian gerbil model is not adequate to evaluate the effect of *H. pylori*-associated gastric inflammation followed by development of GC [46]. This assumption warrants a more thorough discussion.

H. pylori Infection and Carcinogenesis

Recently, epigenetic silencing of gene expression by CpG island methylation was recognized as an important

mechanism in inactivating tumor suppressor genes [47]. Leung et al. and Chan et al. reported that promoter methylation in E-cadherin was frequently detected in the stomach of *H. pylori*-infected patients. Eradication of *H. pylori* possibly reduced the methylation density in the E-cadherin gene and the chance of subsequent neoplastic transformation [48,49]. Another important factor for carcinogenesis might be gene mutations. Yao et al. reported that *H. pylori* might lead to an accumulation of genomic mutations, independently of inflammation. This is associated with a reduced DNA mismatch repair, and is in part associated with CpG methylation of the hMLH1 promoter [50]. In clinical samples, Watari et al. reported that K-ras mutations occurred at an early stage, and are influenced by *H. pylori* infection, and that some mutations may also be selected by eradication [51].

Gastric cancers express enhanced levels of MMPs and their tissue inhibitors (TIMPs). MMP-7 is produced in epithelia and increases with *H. pylori* infection. McCaig et al. studied the role of MMP-7 in signaling between epithelial cells and a stromal cell type, myofibroblasts, and found that MMP-7 from *H. pylori*-infected epithelial cell medium stimulated proliferation and migration of gastric myofibroblasts. Proliferation of gastric epithelial cells was also stimulated by MMP-7-treated myofibroblasts [52]. These results suggest that MMP-7 is a critical regulator for redefining the gastric microenvironment and leads to hyperproliferation in response to *H. pylori* infection. An elevated expression of tissue matrix metalloproteinase 1 (MMP-1) is also associated with GC. Wu et al. investigated the regulation of MMP-1 expression during *H. pylori* infection and found increased MMP-1 mRNA levels in the gastric mucosa and epithelial cells [53].

A basic and important concept concerning the pathogenesis of *H. pylori* was reported by Necchi et al. It is not clear how *H. pylori*, an apparently extracellular pathogen, colonizes the luminal side of the gastric epithelium. They showed that *H. pylori* penetrates normal, metaplastic, and neoplastic gastric epithelium in vivo, intracellularly or interstitially, causing a strong immune-inflammatory response and promoting gastric carcinogenesis [54].

Conflicts of interest

The authors have declared no conflicts of interest.

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A Review of the Postulated Mechanisms Concerning the Association of *Helicobacter pylori* with Ischemic Heart Disease

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Helicobacter pylori, CagA, ischemic heart disease, atherosclerosis, lipids, inflammation, coagulation, homocysteine, endothelial dysfunction.

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Abstract

Since its discovery, *Helicobacter pylori* has been implicated in the pathogenesis of several diseases, both digestive and extradigestive. Interestingly, the majority of the extradigestive-related literature is focused on two vascular manifestations: stroke and ischemic heart disease. Potential mechanisms for the establishment of a *H. pylori*-induced ischemic heart disease have been proposed with regard to chronic inflammation, molecular mimicry, oxidative modifications, endothelial dysfunction, direct effect of the microorganism on atherosclerotic plaques as well as changes regarding traditional or novel risk factors for ischemic heart disease or even platelet-*H. pylori* interactions. A positive link between *H. pylori* infection and ischemic heart disease has been suggested by a series of studies focusing on epidemiologic evidence, dyslipidemic alterations, upregulation of inflammatory markers or homocysteine levels, induction of hypercoagulability, oxidation of low-density lipoprotein, causation of impaired endothelial function, detection of *H. pylori* DNA in atherosclerotic plaques, and participation of certain antigens and antibodies in a cross-reactivity model. There are studies, however, which investigated the relationship between *H. pylori* and ischemic heart disease with regard to the same parameters and failed to confirm the suggested positive association. Further studies in the direction of interaction between *H. pylori* and the host's genotype as well as a quest for evidence towards novel risk factors for ischemic heart disease such as oxidative stress, vascular remodeling, vascular calcification, or vasomotor activity, may reveal a field of great interest, thus contributing to the determination of new potential mechanisms.

The possible link between infectious agents and atherosclerotic process, leading to ischemic heart disease (IHD), has been debated for more than a hundred years [1–3]. As soon as Sohal et al. reported in 1968 that infection with Coxsackie B4 virus could cause coronary arteritis, this hypothesis began to gain ground [4]. Since then a similar role has been proposed for other viruses, followed by reports on the detection of herpes simplex virus (HSV) and cytomegalovirus (CMV) [5] in coronary artery samples and the early development of allograft arteriosclerosis, in CMV-infected recipients [6]. As far as bacteria are concerned, a number of studies have correlated seropositivity to *Chlamydia pneumoniae* with myocardial infarction (MI) while others have shown the presence of *C. pneumoniae* in coronary atheromas [7–12]. From the same bacterial standpoint a number of dental microbes [13,14] and *Helicobacter pylori* have also been proposed as possible contributors to the development of IHD, yet the results from relevant studies proved to be quite contradictory.

H. pylori's Link to Digestive and Extradigestive Diseases. Postulated Causative Mechanisms for *H. pylori*-Induced IHD

H. pylori's role in the development of gastritis and peptic ulcer disease has been highlighted over the past two decades. Along with this well-supported causality came the discovery that mucosa-associated lymphoid tissue (MALT) lymphoma [15,16] and gastric cancer [17,18] are strongly associated with the presence of *H. pylori* in the gastric mucosa. Idiopathic achalasia [19], celiac disease [20], colorectal neoplasia [21], gallstone disease [22,23], primary biliary cirrhosis, and primary sclerosing cholangitis

Table 1 Postulated mechanisms for the onset of a *H. pylori*-induced ischemic heart disease

1. *H. pylori* induction of an atherogenic modified lipid profile
2. Systemic increase of inflammatory markers and mediators
3. Establishment of a hypercoagulable state
4. Molecular mimicry
5. Oxidative modification, which contributes to atherogenesis
6. Changes regarding homocysteine levels
7. *H. pylori* induction of endothelial dysfunction and increase of vasoconstrictor factors
8. Direct effect of *H. pylori* on the progression and instability of atherosclerotic plaques
9. *H. pylori* induction of platelet aggregation

[24] are also examples of *H. pylori*'s assumed involvement in the pathogenesis of diseases affecting the digestive system. In addition to the reports implicating *H. pylori* in the development of digestive diseases, there are several studies that imply a possible relationship between *H. pylori* infection and a number of extradigestive diseases [25]. Although the list is quite long, most reports in the literature are focused on two specific vascular diseases: stroke [26] and IHD. Notwithstanding the number of studies published regarding *H. pylori* infection and IHD, the relationship between the former and the latter still remains in dispute and so does the way in which *H. pylori* could induce or accelerate atherosclerosis in the coronary arteries, leading to IHD. The postulated mechanisms for the onset of a *H. pylori*-induced IHD are showed in Table 1.

The quest for evidence that could support one or more of the hypotheses led to the publication of contradictory results, which have made the association of *H. pylori* infection with IHD quite foggy. In the present review, an effort has been made so that eligible studies offering similar results are presented together, classified into categories according to the provided evidence.

Epidemiologic Evidence

The Case in Favor

In 1994 Mendall et al. reported a higher prevalence of *H. pylori* infection among patients suffering from IHD [27]. It was not long until Danesh et al. suggested a relationship between IHD and persistent infection with *H. pylori*, *C. pneumoniae* and CMV, based on evidence of seropositivity in patients with IHD [28]. A study carried out by Pellicano et al. also showed a higher prevalence of *H. pylori* infection in patients with IHD (odds ratio [OR]: 2.36, 95% confidence interval [95% CI]: 1.08–5.31) [29]. At the same time, a case control English study on 1122 patients younger than 65 years, suffering from acute myocardial infraction (AMI) and, 1122 age- and sex-matched controls, showed a higher prevalence of *H. pylori* seropositivity in AMI patients, even after adjustment for socioeconomic status (OR: 1.87, 95% CI: 1.42–2.47, $p < .0001$) [30]. These findings were in agreement with evidence of excess *H. pylori* seropositivity in patients who had suffered [31,32] or died of MI ($p = .039$) [33], even after multivariate analysis in which confounding factors such as age, sex, smoking, and hypertension were included (OR: 1.35, 95% CI: 1.01–1.83) [31]. A higher seroprevalence, based on anti-*H. pylori* IgG determination, has also been reported in patients with unstable angina (OR: 3.82, 95% CI: 1.27–12.04) [34]. Fischbacher et al. showed that this higher seroprevalence of *H. pylori* in patients with IHD was observed in UK South Asian (OR: 1.79, 95% CI: 1.01–3.17) but not in UK European populations [35], while Gillum associated *H. pylori* infection with IHD only in men with diabetes [36]. In a more recent study, Vijayvergiya et al. showed that there is a relationship between *H. pylori* IgG seropositivity and coronary artery disease [37]. Premature MI has been associated with cytotoxin-associated gene A (CagA) bearing strains of *H. pylori* in a study performed by Gunn et al. ($p = .01$ in subjects < 55 years old) [38], while in other studies IHD correlated with CagA-positive strains [39–41]. This association remained significant even after adjustment for socioeconomic status, body mass index (BMI), diabetes mellitus, hypertension, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol levels and treatment with statins (OR: 1.51, 95% CI: 1.06–2.16) [42].

The Case Against

There are studies, however, whose results are not concordant with the ones mentioned above. In a meta-analysis of five prospective studies performed by Danesh, comparison of *H. pylori*-seropositive patients with *H. pylori*-seronegative individuals yielded a combined risk ratio for IHD of 1.13 (95% CI: 0.93–1.38) [43]. McDonagh et al. also failed to show any significant increase in the OR in *H. pylori*-seropositive individuals with regard to any manifestation of IHD [44]. Since then a number of studies did not show any positive correlation between serologically [45–47] or histologically [48–50] documented *H. pylori* infection and the risk for IHD [51–53]. Zhu et al. based on the results from a cross-sectional and a longitudinal study suggested that *H. pylori* infection could not lead to IHD or AMI (hazard ratio [HR]: 1.12, 95% CI: 0.81–1.54) [54]. Other studies showed no significant association between the titer of antibodies against *H. pylori* and the risk for MI [55,56]. Heider et al. reported that anti-*H. pylori* IgG seropositivity is not associated with incident cardiovascular disease in a

mean follow-up period of 10 years (HR: 1.09, 95% CI: 0.81–1.46). The calculated HR and 95% CI after adjustment for age, gender, BMI, diabetes mellitus, hypertension, total cholesterol, and HDL cholesterol levels were 1.02 and 0.75–1.4, respectively [57]. Coles et al. found no association between serum anti-*H. pylori* IgG levels and the risk for developing IHD or stroke [58]. This lack of association between *H. pylori* infection and IHD has also been suggested in a study by Schiele et al., who did not find a high risk for restenosis after percutaneous transluminal coronary angioplasty (PTCA), in *H. pylori*-infected patients [59]. Moreover, *H. pylori* seropositivity has been associated with a lower occurrence of venous bypass graft occlusion in patients with IHD who had undergone bypass surgery, since the rate of seropositivity in the occlusion-free group was higher than in the group with occlusion ($p = .004$) [60].

Even the seropositivity for CagA-bearing strains of *H. pylori* has been shown to be unrelated to any IHD-associated manifestations. In 1999, Koenig et al. reported a similar prevalence of CagA-bearing strains between patients with IHD and healthy controls ($p = .076$) [61]. A year later, the results of the above-mentioned study had been confirmed by a case control study of 505 patients with IHD and 1025 controls in which the OR for IHD after adjustment was 1.1 (95% CI = 0.71–1.71), when CagA-seropositive individuals were compared with *H. pylori*-seronegative individuals [62]. Murray et al. also reported similar results regarding the association between CagA-positive strains and the risk for MI (OR = 1.16, 95% CI = 0.79–1.7) after adjustment for age, sex, number of siblings, smoking, and socioeconomic status [63]. A prospective study by Stone et al. showed no evidence of a positive association between anti-CagA seropositivity and either incident IHD (adjusted OR = 1.18, 95% CI = 0.76–1.85) or death from IHD (adjusted OR = 1.13, 95% CI = 0.61–2.07) [64]. This result was in agreement with the one described in a case control study of 223 AMI patients and 223 healthy controls who were all studied in relation with anti-CagA seropositivity. Anti-CagA antibodies were present in 33.8% of the cases and in 26.8% of the control subjects yielding an OR and a 95% CI of 1.4 and 0.84–2.33, respectively [65].

Studies with Focus on Lipids

The Case in Favor

In 1995 Murray et al. reported lower HDL cholesterol values in *H. pylori*-infected women ($p = .006$) [66]. A similar observation was made by Niemelä et al., who found that in *H. pylori*-infected individuals, HDL cholesterol concentrations were lower, as opposed to the triglycerides concentrations that were higher [67]. Elevated serum triglyceride levels were also reported in another study group consisting of 460 men, who were seropositive for both anti-*H. pylori* IgG and anti-*H. pylori* IgA, along with higher total cholesterol concentrations and a lower HDL/total cholesterol ratio. These associations remained significant in non-smokers even after adjustment for age, BMI, and social class: $p = .0014$ for triglycerides, $p = .003$ for total cholesterol, and $p = .013$ for HDL/total cholesterol ratio [68]. In another study by Hoffmeister et al. it has been shown that *H. pylori* infection correlated with lower HDL values ($p = .002$), a lower HDL/total cholesterol ratio ($p = .005$), lower apolipoprotein AI (ApoAI) concentrations ($p = .02$), and higher apolipoprotein B concentrations ($p = .03$) [69]. The association between infection with *H. pylori* and an altered lipid profile remained significant in a group of *H. pylori*-infected diabetic patients whose HDL-cholesterol levels proved to be lower, in contrast to the triglyceride levels which were higher ($p < .001$ for both the HDL and the triglycerides) [70]. In another study by deLuis et al., HDL concentrations increased while lipoprotein a -Lp(a)- concentrations decreased after successful eradication of *H. pylori* in patients with type I diabetes [71]. Moreover, Majka et al. reported that higher levels of LDL and total cholesterol, at baseline, in *H. pylori*-infected individuals decreased after a successful eradication treatment [72]. Eradication treatment for *H. pylori* also led to a statistically significant ($p < .001$) increase in HDL cholesterol, ApoAI, and ApoAII in a study by Schamagl et al. [73], and this result was in accordance with a report by Kanbay et al., who observed an increase in HDL concentrations after *H. pylori* eradication [74].

The Case Against

All the above-mentioned studies implicate *H. pylori* infection into causing an atherogenic modified lipid profile which in turn could lead to IHD. There are studies, however, whose results do not support the idea of an *H. pylori*-dependent dyslipidemia. Patel et al. found no association between *H. pylori* infection and levels of total cholesterol and triglycerides [75]. This lack of association between *H. pylori* and serum lipids was confirmed by two more studies [76,77]. The three above-mentioned studies were included in a meta-analysis of 18 studies, performed by Danesh et al. [78]. The results provided by the meta-analysis showed no correlation between *H. pylori* infection and the concentrations of total cholesterol or triglycerides. A lack of association between *H. pylori* and HDL-cholesterol or triglyceride levels has been reported in a study by Gillum, in a group of men, aged 40–74 years [36]. In a prospective study of 618 Japanese patients with AMI and 967 controls, Kinjo et al. found no detrimental effect of

H. pylori infection on the classic coronary risk factors including total and HDL cholesterol [79]. Similar results were reported in another study on a Greek population [80].

Even the changes in serum lipid concentration after eradication have been challenged by a number of studies. Elizalde et al. studied a population of 686 *H. pylori*-positive patients, in relation with lipid levels. As reported in this study, infection with *H. pylori* had no influence on baseline lipid levels [81]. The changes in triglyceride, HDL, LDL, and total cholesterol levels also remained nonsignificant after eradication of *H. pylori*, in another study by Lu et al. [82], thus making the association between *H. pylori* infection and lipid modifications weaker.

Studies with Focus on Markers of Inflammation

The Case in Favor

It is suggested that a chronic low-grade inflammatory process leading to atherosclerosis could be a possible mechanism for the onset of an *H. pylori*-induced IHD. This hypothesis has gained more ground since evidence of increased concentrations of markers of inflammation as a result of *H. pylori* infection has been reported. Elevated concentrations of interleukin-6 (IL-6), tumor necrosis factor-a (TNF-a), IL-1b, and IL-8 in the gastric mucosa have been reported in five different studies [83–87]. Higher levels of circulating TNF-a have been found in *H. pylori*-infected individuals, in a study by Russo et al. too [88]. Moreover, the levels of circulating TNF-a, IL-1b, and IL-8 decreased significantly after successful eradication of *H. pylori*, in a group of patients who had undergone PTCA [89]. Consolazio et al. also reported higher baseline levels of circulating TNF-a in patients with chronic *H. pylori*-positive gastritis. These higher TNF-a titers fell significantly after successful eradication, reaching levels observed in *H. pylori*-negative patients [90]. The assessment of adhesive molecules in the sera of children with active *H. pylori* infection has shown the presence of higher soluble vascular cell adhesion molecule-1 (sVCAM-1) levels [91], which has been shown to correlate to the presence and severity of IHD [92].

The association between infection with *H. pylori* and IHD has also been investigated with focus on white blood cell count (WBC) and C-reactive protein (CRP). Pieniazek et al. reported higher levels of CRP in *H. pylori*-infected patients with IHD than in controls [40]. A significant positive correlation between the anti-*H. pylori* IgG titer and CRP has been observed in a group of patients with vasospastic angina in a study performed by Hara et al. [93]. In another study by Kanbay et al., CRP levels decreased after successful *H. pylori* eradication treatment [74].

The Case Against

There are studies, however, who failed to confirm the suggested positive correlation between *H. pylori* infection and CRP levels. Two separate studies performed by Abdelmouttaleb et al. [94] and Roivainen et al. [55] failed to show any association between the levels of CRP and infection with *H. pylori*. Brenner et al. also suggested that *H. pylori* infection is unrelated to CRP levels and the leukocyte count in healthy adults [95]. This has been confirmed by Cook et al. in a population of children [96]. Koenig et al. also failed to associate CRP and leukocyte levels with *H. pylori* infection [61] and so did Danesh et al. [97]. A lack of such an association has been shown by Singh et al. in a case control study of individuals who had a coronary event during the follow-up period, and healthy controls. They observed that positivity for CagA-bearing strains did not correlate with any significant increase in CRP or WBC, both in patients and in controls [42]. Stone et al. reported that the treatment of patients, who had experienced AMI or unstable angina, with amoxicillin, metronidazole, and omeprazole led to a significant fall in the CRP levels, but this beneficial effect proved to be independent of *H. pylori* seropositivity [64]. Moreover, Delanghe et al. showed that there was no association between infection with *H. pylori* and levels of CRP, serum amyloid A, or serum haptoglobin [98]. Finally, the Third National Health and Nutrition Examination Survey showed that *H. pylori* infection did not correlate with serum CRP [36].

Effect of Infection with *H. pylori* on Coagulation Parameters

The Case in Favor

The hypothesis that *H. pylori* infection could induce changes in coagulation parameters has been investigated in a number of studies with focus on fibrinogen, prothrombin fragments, plasminogen-activating inhibitor 1 (PAI-1), factor VII, and even von Willebrand factor (vWF). Patel et al., in a cross-sectional study, found an association between *H. pylori* seropositivity and fibrinogen levels in patients with IHD [75]. Basili et al. also showed that patients with *H. pylori*-positive gastritis had greater values of fibrinogen, compared to *H. pylori*-negative controls ($p = .0004$) [99]. These higher levels of fibrinogen fell significantly after treatment with antibiotics in a study by Torgano et al. [100]. Successful eradication of *H. pylori* led to a decrease in fibrinogen levels in two different studies performed on patients with symptomatic IHD [101] or stroke [72]. It has also been reported that individuals with *H. pylori*-positive gastritis have higher levels of prothrombin

fragment 1+2, compared with *H. pylori*-negative gastritis patients and *H. pylori*-negative individuals with a normal gastric mucosa ($p < .05$). Moreover, the prothrombin fragment 1+2 levels fell significantly during a period of 2 months, following successful eradication of *H. pylori* ($p = .03$) [90]. Elizalde et al. have demonstrated that *H. pylori* infection in mice induces the formation of platelet aggregates [102]. By damaging the mesenteric arteriole endothelium by laser pulses, another group was able to show a significant increase in the number of platelet emboli and in the duration of embolization in mice chronically infected with *H. pylori* as compared to non-infected mice [103]. Thus this experimental evidence supports the concept that the role of the bacterium could be even more important in the acute event. In another study designed and carried out by Byrne et al., it has been reported that some strains of *H. pylori* bind vWF and induce platelet aggregation via interaction with glucose phosphate isomerase-b (GPIb) [104].

The Case Against

Other studies failed to link *H. pylori* infection with the presence of a hypercoagulable state. Murray et al. found a weak negative association between *H. pylori* infection and fibrinogen levels ($p = .02$) [66], while Ossei-Gerning et al. reported no association between *H. pylori* serostatus and circulating levels of fibrinogen, PAI-1, vWF, and factor VII in patients undergoing coronary angiography [105]. Wald et al. also failed to show any significant difference in the concentration of fibrinogen between *H. pylori*-positive and *H. pylori*-negative individuals [77]. Koenig et al. did not show any correlation between *H. pylori* infection and either fibrinogen levels or plasma viscosity [61]. A lack of a significant association between seropositivity for *H. pylori* and either fibrinogen or prothrombin cleavage fragments has been shown in the study of Parente et al. [106]. De Backer et al. showed that fibrinogen levels did not correlate with *H. pylori* antibody levels [107], while Singh et al. reported no association between positivity for CagA-bearing strains and any significant changes in fibrinogen values among patients with IHD and controls [42]. Even the decrease in fibrinogen levels after eradication treatment has been denied by a number of studies. Schweeger et al. failed to confirm any significant fall regarding the levels of fibrinogen after successful *H. pylori* eradication [108], while Stone et al. showed that the decrease in fibrinogen levels, following the administration of antibiotics, was independent of *H. pylori* seropositivity [64]. Lu et al. found no changes in fibrinolytic profiles including tissue plasminogen activator (t-PA), PAI-1, fibrinogen, and D-dimers after successful eradication in *H. pylori*-infected individuals [82]. Finally, Elizalde et al. reported that neither baseline *H. pylori* infection nor *H. pylori* eradication in baseline *H. pylori*-infected cases was associated with any significant variations in platelet activation markers such as P-selectin or platelet surface expression of CD62P, CD63 and CD41 [109].

H. pylori Infection and Variations in Homocysteine Concentration

Studies Recording Variations

It has been proposed that infection with *H. pylori* could lead to an elevation of homocysteine titers as a result of reduced folate [110] and/or poor B12 absorption [111]. This hypothesis is mainly supported by the study of Tamura et al., who reported lower serum levels of vitamin B_{12} ($p = .02$) and folate ($p = .046$) as well as higher homocysteine levels ($p = .01$) in *H. pylori*-infected subjects compared to individuals with a negative *H. pylori* histology [112].

Studies Recording No Variations

The vast majority of the associated literature, however, supports the case against. Saxena et al. [113] and Whincup et al. [62] in two separate studies did not find any significant difference in homocysteine levels between *H. pylori*-seropositive and seronegative individuals ($p = .30$ and $p = .98$, respectively). Yoshino et al. reported similar results ($p = .63$) [114]. The difference in serum homocysteine levels between *H. pylori*-infected and uninfected subjects remained nonsignificant ($p = .16$) in the study of Leung et al. [115]. This result was in agreement with the report of Kowalski that *H. pylori* eradication had no effect on homocysteine levels in patients who had undergone PTCA [89].

H. pylori Detection in Atherosclerotic Plaques

Positive Results

The *H. pylori*'s implication in the process of atherogenesis has been investigated by means of polymerase chain reaction (PCR) techniques, aiming at a potential detection of *H. pylori* DNA in atheromatic lesions. Farsak et al. studied 46 endarterectomy specimens obtained from atherosclerotic lesions, serving as cases, and 39 samples excised from healthy regions of the ascending aorta, accepted as controls. *H. pylori* DNA was found via PCR in 17 of the 46 cases and none of the controls ($p < .001$) [116]. Kowalski et al. in two different studies reported that 22 of 46 IHD patients were tested positive for *H. pylori* DNA in atherosclerotic

plaques, whereas none of the 19 controls showed evidence of *H. pylori* DNA. Moreover, 11 of 14 anti-CagA positive patients from the IHD group showed positive detection of *H. pylori* DNA ($p = .015$) [89,117]. A correlation between DNA presence and prior MI ($p = .008$) and unstable angina ($P < 0.001$) was also found in the IHD group [117]. Amerisо et al. found *H. pylori* DNA in 20 of 38 atherosclerotic plaques, and reported immunohistochemical evidence of *H. pylori* infection in 10 of these 20 *H. pylori* DNA-positive plaques [118]. In another study by Kaplan et al., *H. pylori* DNA was detected in nine of 52 atheromatic specimens, but in none of the macroscopically healthy ascending aorta wall specimens ($p = .003$) [119]. In two different studies, Rassu et al. found *H. pylori* DNA in four of 18 atheroma specimens [120], while Adiloglu et al. reported evidence of *H. pylori* DNA in three of 14 coronary endarterectomy atherosclerotic specimens and in one of the 15 left internal mammary artery specimens [121].

Negative Results

The presence of *H. pylori* genomic material in atherosclerotic lesions shown in the above-mentioned studies has not been verified by the results reported by Danesh et al. as they detected *H. pylori* DNA in only one of the 39 carotid atheroma specimens [122]. No *H. pylori* DNA was traced in atherosclerotic plaques obtained by endarterectomy, in another study by Sulewska et al. [123]. Finally, in a recent study, Kaklikkaya et al. reported no evidence of *H. pylori* DNA in aortoiliac atherectomy specimens taken from 21 patients with aortoiliac occlusive disease [124].

Studies with Focus on Postulated Cross-Reactivity

In Favor of Cross-Reactivity

Based on the hypothesis that an immune response mounted against antigens on *H. pylori* can cross-react with homologous host protein in a form of molecular mimicry, some authors investigated the role of heat-shock proteins (hsp) that are expressed by *H. pylori* and are also present in humans. The presence of antibodies against hsp60 and antimycobacterial hsp 65 (mHSP65) has been shown to correlate with atherosclerosis in carotid [125] and coronary arteries [126]. Birnie et al. recruited 100 *H. pylori*-infected patients and found that successful eradication led to a significant fall in anti-hsp65 titers ($p = .033$) [127]. Xu et al. managed to correlate elevated serum soluble hsp 60 levels with *H. pylori* infection [128]. An association between serum antibodies to mHSP65 and seropositivity to *H. pylori* has been reported in two different studies by Mayr et al. ($p = .001$) [129] and Prohaszka et al. [130]. Moreover, mHSP65 antibody titers have been shown to correlate with *H. pylori* infection ($p = .004$). This correlation maintained significance after adjustment for IHD risk factors and seropositivities to other pathogens (adjusted OR: 3.1, 95% CI: 1.4–6.6) [131]. An association between mHSP65 antibodies and elevated coronary calcification levels, independent of IHD risk factors after multivariate adjustment ($p = .037$), has also been reported in this study. Anti-CagA antibodies have been shown to react with cytoplasm and nuclei of smooth muscle cells, cytoplasm of fibroblast-like cells, and the cell membranes of endothelial cells in the study of Franceschi et al. Anti-CagA antibodies also specifically immunoprecipitated two high molecular weight antigens of 160 and 180 kDa from artery lysates [132].

Against Cross-reactivity

Rothenbacher et al. did not find any significant association between antihsp60 antibodies, expressed as hsp60 extinction, and IHD (OR = 1.28, 95% CI: 0.90–1.81) [133].

Other Studies

According to the oxidative modification hypothesis of atherosclerosis, LDL oxidation is an early event in atherosclerosis, and oxidized LDL (ox-LDL) contributes to atherogenesis [134]. The potential role of *H. pylori* infection in the induction of changes regarding the levels of ox-LDL has been investigated by Kayo et al., who found no association between plasma levels of ox-LDL and *H. pylori* infection [135]. Chang et al. on the other hand, have managed to show that successful eradication of *H. pylori* led to a significant ($p < .01$) decrease in the levels of endothelin-1, and a significant ($p < .05$) elevation of nitrate/nitrite levels [136]. The possible role of *H. pylori* in the induction of endothelial dysfunction has also been studied by Prasad et al. [137]. According to their findings, microvascular and epicardial dilation with acetylcholine tended to be lower in subjects who were seropositive for *H. pylori* ($p = .002$).

Conclusions – Directions for Future Studies

As already shown above, the results originating from the studies investigating the relationship between infection with *H. pylori* and IHD, with focus on many different risk factors proved to be quite contradictory, thus making it difficult to determine whether there is a link between this microorganism and IHD or not. Nevertheless, the presence of *H. pylori* DNA in atherosclerotic lesions, the presence of antibodies against hsps in patients with IHD, along with the already described cross-reactivity of antibodies against

H. pylori with vascular components is evidence that should not be taken lightly. Rechcinski et al., based on the results from two different studies, proposed the intensive humoral response to *H. pylori* antigens as a causative mechanism for atherosclerosis [138,139]. Grebowska et al. confirmed this intense humoral response and also implicated the host's predisposition to respond to Lewis determinants that are present in *H. pylori* lipopolysaccharide (LPS) with the production of IgG, in the atherogenic process [140]. The genetic susceptibility of the host has been highlighted in the study of Candore et al., who demonstrated that a specific mutation regarding Toll-like receptor 4 (TLR4) shows a significantly lower frequency in patients affected by MI, compared to controls [141]. This TLR4 ASP299GLY polymorphism is associated with a weaker innate immune response and lower IL-6 levels, which means that people who share this specific polymorphism, seem to have fewer chances of developing cardiovascular diseases. Perhaps further studies, in the direction of interaction between *H. pylori* and the host's genotype which in turn could determine the type and intensity of the immune-inflammatory responses held accountable for the induction of IHD, could clarify this matter. A quest for evidence towards novel risk factors for IHD such as oxidative stress markers, renin-angiotensin system participants, markers of endothelial dependent vasodilation–vasoconstriction, enzymes, or other proteins participating in the vascular remodeling with regard to *H. pylori* infection, might also prove useful. Since an association between mHSP65 antibodies and elevated coronary artery calcification has already been reported [131], the determination of coronary artery calcium by means of electron beam computed tomography in *H. pylori*-infected IHD patients might reveal a field of great interest and encourage the study of calcification-related parameters [142]. Thus, it seems that the concept of *H. pylori*'s implication in the pathogenesis of IHD is still open for discussion and needs to be studied so that a widely accepted mechanism through which *H. pylori* infection can result in IHD could be determined.

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Strategy To Characterize the Number and Type of Repeating EPIYA Phosphorylation Motifs in the Carboxyl Terminus of CagA Protein in *Helicobacter pylori* Clinical Isolates[▽†]

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Cytotoxin-associated gene A (CagA) diversity with regard to EPIYA-A, -B, -C, or -D phosphorylation motifs may play an important role in *Helicobacter pylori* pathogenesis, and therefore determination of these motifs in *H. pylori* clinical isolates can become a useful prognostic tool. We propose a strategy for the accurate determination of CagA EPIYA motifs in clinical strains, based upon one-step PCR amplification using primers that flank the EPIYA coding region. We thus analyzed 135 *H. pylori* isolates derived from 75 adults and 60 children Greek patients. A total of 34 cases were found to be EPIYA PCR negative and were consequently verified as *cagA* negative by *cagA*-specific PCR, empty-site *cagA* PCR, and Western blotting. Sequencing of the remaining 101 PCR-positive amplicons confirmed that an accurate prediction of the number of EPIYA motifs on the basis of size distribution of the PCR products was feasible in all cases. Furthermore, our assay could identify closely related *H. pylori* subclones within the same patient, harboring different numbers of EPIYA repeats. The prevalence of CagA proteins with three EPIYA motifs (ABC) or four EPIYA motifs (ABCC) was the same within the adult and children groups. However, CagA species with more than four EPIYA motifs were observed exclusively within adults (8.6%), suggesting that CagA-positive strains may acquire additional EPIYA-C motifs throughout adulthood. Our strategy requires no initial *cagA* screening of the clinical isolates and can accurately predict the number of EPIYA repeats in single or multiple closely related subclones bearing different numbers of EPIYA motifs in their CagA, which may coexist within the same patient.

CagA, a 120- to 145-kDa bacterial protein, is recognized as a major etiologic determinant of *Helicobacter pylori*-associated gastric disease found to increase the risk for peptic ulceration (14, 15, 23), atrophic gastritis (20) and non-cardia gastric adenocarcinoma (12, 25). After *H. pylori* binding to the gastric epithelium, CagA has been shown to translocate into the gastric epithelial cell cytoplasm via the *H. pylori* type IV secretion system (24). Once injected into the epithelial cells, CagA localizes to the plasma membrane (3) and undergoes tyrosine phosphorylation (27) by multiple members of the Src family of kinases (30, 28) on specific tyrosine residues within repeating Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs (9, 17). These EPIYA motifs are defined as EPIYA-A, -B -C, and -D, according to the amino acid sequence that surrounds the EPIYA sequence (17). Earlier studies have showed that CagA protein species nearly always contain EPIYA-A and EPIYA-B sites, followed by one to three repeats of EPIYA-C in Western-type *H. pylori* isolates (26) or EPIYA-D site in East Asian-type isolates (8, 35, 36). Thus, CagA species usually vary on the number of EPIYA-C or -D repeats in the carboxyl terminus of the protein. Phosphorylated CagA has been reported to interact with and deregulate the activity of a number of intracellular effectors relating to the hepatocyte growth factor signaling pathway, such as Src homology 2-containing protein tyrosine phosphatase 2 (16), growth factor receptor bound 2 (21), carboxyl-terminal Src kinase (29), and hepatocyte growth factor receptor/cMet (13). More specifically, tyrosine-phosphorylated CagA seems to bind and deregulate the activity of Src homology 2-containing protein tyrosine phosphatase 2 via the Western CagA-specific EPIYA-C or East Asian CagA-specific EPIYA-D site and of carboxyl-terminal Src kinase via the EPIYA-A or EPIYA-B site (22). In parallel, CagA EPIYA motifs have been suggested to play an essential role for the tethering of CagA to the membrane in a phosphorylation-independent manner (18). Consequently, CagA variability with reference to EPIYA motifs may play an important role in *H. pylori* pathogenesis. CagA-positive clinical strains with an increased number of EPIYA phosphorylation motifs isolated from Eastern populations have been associated with more severe active chronic gastritis and atrophy (8). An increasing number of EPIYA motifs within the Western-type CagA have been related with higher interleukin-8 secretion and more pronounced cellular elongation (6). Therefore, EPIYA motif diversity may prove useful in the prediction of *H. pylori* pathogenic activity, and the accurate determination of the type and number of EPIYA motifs in clinical *H. pylori* isolates can become a useful prognostic tool.

In a clinical microbiology laboratory, *H. pylori* isolation from

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gastric biopsies is often made by the sweeping method instead of individual colony selection and, therefore, the presence of multiple closely related subclones within an *H. pylori* isolate is likely to occur (10, 11). Such *H. pylori* subclones harboring different numbers of CagA EPIYA motifs have been observed to coexist within the same patient (5). An elegant PCR-based assay for the determination of CagA EPIYA motifs, utilizing three separate sets of primers specific for each EPIYA motif, has already been proposed (7). However, it assumes clonal uniformity with reference to EPIYA motif diversity within an isolate. Hence, during routine screening of *H. pylori* clinical isolates, the use of such a method for the determination of EPIYA motifs may produce ambiguous results due to the presence of closely related subclones with different numbers of CagA EPIYA motifs.

In the present study, we designed specific primers and successfully amplified the variable 3′ end of *cagA* gene and then sequenced the PCR products in more than 100 *H. pylori* clinical strains. Based on our sequencing results, the numbers and types of EPIYA motifs within CagA protein can safely be predicted by the size of the one-step PCR amplicon in more than 90% of the cases. We also confirmed that our method could accurately predict *cagA* presence in the isolated strains, by *cagA*-specific PCR, empty-site *cagA* PCR, and Western blot analysis of CagA expression, thus eliminating the need for initial screening of *cagA* status after strain isolation. Furthermore, our strategy enabled us to detect within the same patient the presence of multiple closely related infecting *H. pylori* subclones with different numbers of EPIYA motifs in CagA and facilitated their isolation. In this way, we determined EPIYA diversity within the CagA protein for more than 100 *cagA*-positive clinical *H. pylori* isolates from adults and children.

MATERIALS AND METHODS

Clinical isolates. *H. pylori* clinical isolates ($n = 135$), derived from 75 adult (48 male, 27 female, mean age of 52.1 ± 1.6 years) and 60 child patients (24 male, 36 female, mean age of 9.9 ± 0.6 years) were isolated from gastric biopsies obtained during upper gastroduodenal endoscopy at the Gastroenterology Clinics of Alexandras General Hospital, Evangelismos General Hospital, and Aghia Sophia Children's Hospital. All patients were Greek in origin and signed a consent form for participation in the study. Each clinical isolate was passed twice on Chalgren's-Wilkins agar plates containing antibiotics (vancomycin [10 μg/ml], trimethoprim [10 μg/ml], polymyxin B [10⁴ IU/liter], amphotericin B [2 μg/ml], nalidixic acid [10 μg/ml], bacitracin [30 μg/ml], fluorocytosine [5 μg/ml]; Sigma, St. Louis, MO) supplemented with 7% (vol/vol) horse blood and 1% (vol/vol) Vitox (Oxoid, Basingstoke, United Kingdom). Cultures were incubated at 37°C under microaerophilic conditions (CampyPak-Plus; Becton Dickinson, Cockeysville, MD). The total bacterial genomic DNA was extracted by using the DNeasy isolation kit (QIAGEN AS, Oslo, Norway), and the ratio of the optical density at 260 nm to that at 280 nm was greater than 1.800. *H. pylori* strain SS1 was also included in the study as a control strain since it has a published *cagA* gene sequence. RAPD [random(ly) amplified polymorphic DNA] PCR profiles of isolates were obtained utilizing the primers 1281 and D11344 as described previously (2).

Amplification and sequencing of the EPIYA-containing region of CagA. After initial alignment of full CagA protein sequences found in GenBank, we identified conserved peptide sequences 845′-VKNGVNGTLVGSS-856′ and 1011′-LNQA VSEAK-1019′, which flank the region harboring the EPIYA motifs (positions according to the *H. pylori* 26695 genome [32]). Based upon these peptides we designed the primers cagA2586 (5′-GTTAARAATRGTGTRAAYGG-3′, where R = A or G and Y = T or C) and cagA3000AS (5′-TTTAGCTTCTGA TACCGC-3′; positions 582453 to 582977 with reference to the *H. pylori* 26695 genome). Screening of the oligonucleotides by BLAST analysis identified exclusively *H. pylori cagA* sequences (data not shown).

PCR amplification was carried out within 50-μl reaction mixtures containing 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 1 U of *Taq* (Fermentas UAB, Vilnius, Lithuania), 200 μM concentrations of deoxynucleoside triphosphates, 0.5 μM concentrations of the primers, and 50 ng of microbial genomic DNA. The PCR conditions included an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 50°C for 45 s, and 72°C for 45 s, and then an extension at 72°C for 5 min. The conditions of PCR amplification were optimized by using an Eppendorf Mastercycler gradient apparatus (Eppendorf AG, Hamburg, Germany) with annealing temperatures ranging from 40.0 to 60.4°C (data not shown). Single PCR products ranging from 370 to 670 bp (±25 bp) were initially visualized by agarose gel electrophoresis. They were subsequently isolated by using a QIAquick PCR purification kit (QIAGEN) and sequenced by automated sequencing on a Li-Cor DNA sequencer long read IR2 4200 (IMBB facility, Crete), utilizing IRD700-labeled cagA3000AS primer and a SequiTherm EXCEL II DNA sequencing kit-LC (Epicentre Biotechnologies, Madison, WI). The deduced peptide sequences containing the EPIYA motifs were aligned by CLUSTAL W (European Bioinformatics Institute [http://www.ebi.ac.uk/clustalw/]). During the initial developmental stages of the assay, we used the PCR product of *H. pylori* SS1 strain (accession number AAF63759) as a wire control of a *cagA* nucleotide sequence coding for three EPIYA motifs. All strains were also screened for the presence of the *cagA* gene by PCR as described earlier (33). EPIYA PCR-negative strains were confirmed as being *cagA* negative by the empty site-positive PCR assay for the characterization of *cagA*-negative strains (1).

During initial development stages, we validated the ability of our PCR procedure to amplify the EPIYA-coding region in 61 *cagA*-positive samples by utilizing the primers cag2 and cagI (26), which amplify the variable 3′ region of the *cagA* gene between positions 582471 and 583025 with reference to the *H. pylori* 26695 genome. Finally, based on our sequence data we evaluated the ability of a PCR-based assay reported earlier (7) to accurately predict the number and type of EPIYA motifs.

Analysis of CagA protein expression by Western blot analysis. Human gastric adenocarcinoma epithelial AGS cells, cultured in F-12 Knight'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 under a 5% CO_2 atmosphere for 24 h. Total protein lysates from infected cells or *H. pylori* bacterial preparations were obtained in ice-cold lysis buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.2], 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, 2 mM L-dithiothreitol) containing protease and phosphatase inhibitor cocktails. Lysates were centrifuged at 14,000 × *g* for 30 min at 4°C, and the supernatants were kept at −20°C until use. The total protein was determined by using a Pierce MicroBCA protein assay (Pierce Biotechnology, Inc., Rockford, IL). Equal protein amounts of cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% polyacrylamide) and transferred onto polyvinylidene difluoride (Immobilon-P; Millipore Corp., Bedford, MA) membranes. We performed standard Western blotting with primary anti-CagA monoclonal antibody (Austral Biologicals, San Ramon, CA) at a dilution of 1:1,000 and secondary horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G polyclonal antibody (Jackson Immunoresearch Europe, Ltd., Soham, Cambridgeshire, United Kingdom). CagA expression was detected by utilizing a chemiluminescence detection system (ECL Plus) according to the manufacturer's instructions (Amersham/GE Healthcare UK, Ltd., Buckinghamshire, United Kingdom).

Accession numbers. All partial nucleotide *cagA* sequences generated in the present study have been submitted to the GenBank/EMBL/DDBJ databases (accession numbers AM279268 to AM279335, AM292583 to AM292590, and AM295786 to AM295791).

RESULTS

PCR amplification of the variable 3′ region of the *cagA* gene coding for EPIYA motifs. We successfully amplified the variable 3′ region of the *cagA* gene in 101 of 135 *H. pylori* isolates by utilizing our EPIYA PCR method and observed a single-band PCR product (Fig. 1) in 91 cases and a double-band product (Fig. 2A) in 10 cases. The PCR amplicons ranged in size between 370 and 670 bp (±25 bp) and were arranged equidistantly (approximately 100 bp) in a ladder-like arrangement indicative of the presence of multiple repeated sequences. All strains negative for EPIYA PCR ($n = 34$) were

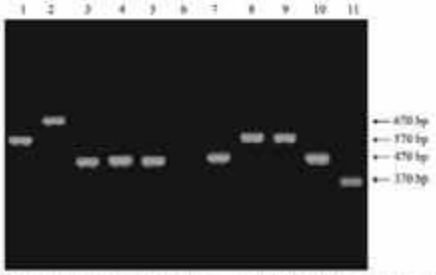

FIG. 1. Electrophoretic analysis of EPIYA PCR products. The DNA from *H. pylori* clinical strains (lanes 1 to 8) was amplified by EPIYA PCR, and the PCR products were analyzed on a 1.5% agarose gel. The samples in lanes 9 to 11 are sequenced EPIYA PCR products from *cagA*-positive strains with ABCC (lane 9; Hp63; AM292557), ABC (lane 10; Hp5; AM292581), and AB (lane 11; Hp51; AM292592) combinations. Note the ladder-like distribution in the molecular weights of the samples, which is indicative of the presence of multiple repeats. Based on their size, the EPIYA number predictions were 3 (samples 3, 4, 5, and 7), 4 (samples 1 and 8), and 5 (sample 2). Sample 6 was a *cagA*-negative isolate.

further verified as true *cagA*-negative strains utilizing a *cagA*-specific PCR, empty-site-positive PCR assay for *cagA*-negative strains and Western blotting. More specifically, using *cagA*-specific PCR we identified only 15 *cagA*-negative strains by the absence of the characteristic 180-bp PCR product (data not shown). However, utilizing the empty-site-positive PCR assay we identified as true *cagA* negative all 34 strains that were EPIYA PCR negative. Finally, no expression of CagA protein was detected by Western blotting in whole-cell bacterial lysates from all EPIYA PCR-negative strains (data not shown). The remaining 101 strains that gave a positive EPIYA PCR product were all confirmed as *cagA* positive by *cagA*-specific PCR and Western blotting of whole-cell bacterial lysates (data not shown). Of these strains, 43 were isolated from children (21 males, 22 females) and 58 were from adult patients (30 male, 19 female). No significant difference in EPIYA PCR positivity was detected between the adult and children populations or between genders. Finally, in 61 strains we also utilized the primers cag2 and cag4 (26), which amplify the EPIYA-coding region in the *cagA* gene between positions 582471 and 583025 with reference to the *H. pylori* 26695 genome. Although three cases were not amplified by the cag2 and cag4 primers, we observed no significant difference ($P = 0.244$ [Fisher's exact test]) in the ability of the two PCR methods to amplify the EPIYA-coding region (data not shown).

EPIYA PCR analysis of the *H. pylori* SS1 strain yielded an amplification product of 470 ± 25 bp, and this was used as a size control for sequences encoding for three EPIYA motifs. Thus, initial prediction on the expected number of EPIYA repeats was possible by direct comparison of the corresponding sizes of PCR amplicons.

In 10 isolates following EPIYA PCR, we detected the presence of a double band with sizes corresponding to different numbers of EPIYA repeats (Fig. 2A). We verified by isolation and sequencing that those were *cagA*-specific sequences, an indication of the presence of at least two infecting strains, within the same patient. 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 (Fig. 2A). We utilized RAPD PCR to assess the clonal relatedness of the isolated subclones and verified that these strains were very closely related to each other (Fig. 3). More specifically, RAPD profiles with primer D11344 were identical between subclones, and those obtained by primer 1281 showed very close relatedness. Collectively these data suggest that our EPIYA PCR assay can (i) accurately predict the presence of the *cagA* gene in *H. pylori* clinical isolates, (ii) efficiently amplify the variable *cagA* gene 3′-region encoding for the EPIYA motifs, and (iii) detect the presence and facilitate the isolation of the individual infecting subclones.

CagA diversity with respect to the number and type of EPIYA motifs. All amplified EPIYA PCR products were sequenced, and the deduced peptide sequences were aligned by using CLUSTAL W (Fig. 4). The *H. pylori* SS1 strain PCR product when sequenced was found to be identical to the already published sequence (accession number AAF63759; and data not shown). Upon alignment of the deduced protein sequences in our clinical samples, we observed three types of EPIYA motifs, namely, (i) EPIYA-A, EPIYAKVNKKK(A/T/V/S)GQ; EPIYA-B, EPIY(A/T)(Q/K)VAKKVNAKI; and EPIYA-C, EPIYATIDDLG (Fig. 4). We found no strains within our population harboring the Eastern type of EPIYA-D (EPIYATIDFDEANQAG). Our initial predictions about the number of EPIYA repeats, based upon the size of the PCR amplicons, were all verified on the basis of the nucleotide and the deduced peptide sequences. Furthermore, in all cases where we isolated from the same patient two closely related subclones predicted to have different number of EPIYA motifs, the amplified nucleotide sequence and the subsequent aligned peptide sequences obtained verified our original prediction (Fig. 2B and C). Upon comparison, sequences were found to be identical on a nucleotide basis, outside the 102-bp sequence repeats coding for the 34-amino-acid peptide segment containing the additional EPIYA-C motifs (Fig. 2B and C). In these closely related subclones, we further verified the expression of the CagA proteins with different numbers of EPIYA motifs by Western blot analysis of total protein lysates, after infection of AGS gastric adenocarcinoma epithelial cells, with the mixed isolates, as well as the individual isolated subclones (Fig. 2D). These data suggest that our EPIYA PCR can effectively identify the number of EPIYA motifs within the CagA protein in *H. pylori* isolates both in single and in multiple closely related subclones bearing different numbers of EPIYA motifs in their CagA proteins.

Comparison of PCR methods for determination of the number of EPIYA motifs. An elegant PCR method utilizing three different sets of primers specific for each EPIYA type coding sequence had been proposed for the determination of the number of EPIYA motifs (7). Having obtained the sequences for the variable 3′ region of *cagA* gene, we compared that PCR method and our EPIYA PCR with regard to their ability to predict the correct number of EPIYA motifs present in 31 single-clone *H. pylori* isolates (Table 1; also see Fig. S1 in the supplemental material). No significant difference was observed between the two methods in predicting three (ABC combination) or four (ABCC combination) EPIYA repeats. However,

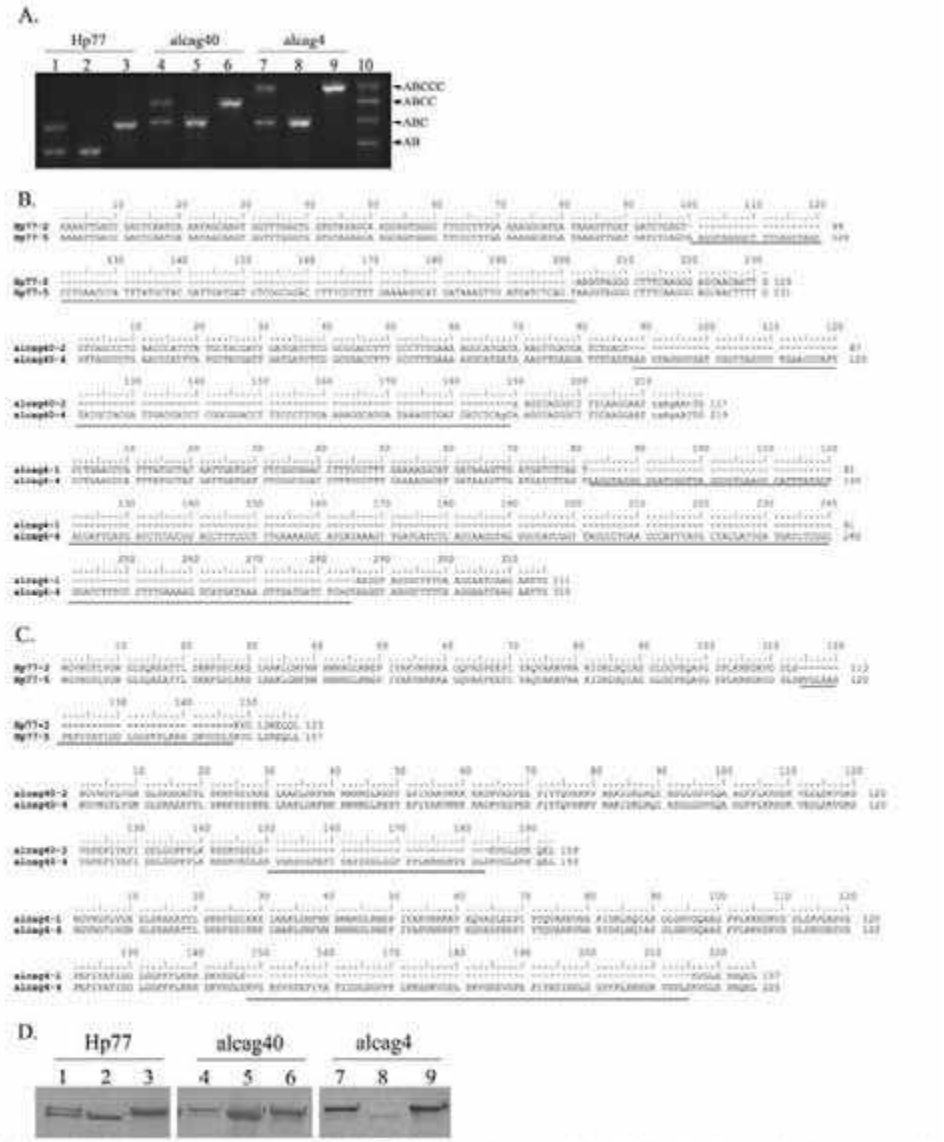

FIG. 2. *H. pylori* closely related paired subclones Hp77-2–Hp77-5, alcag4-1–alcag4-4, and alcag40-2–alcag40-4 isolated from the same respective patients, differing in the number of repeating EPIYA-C motifs. (A) Agarose gel mobilities of EPIYA PCR amplicons in the mixed isolates (lanes 1, 4, and 7) and the respective isolated paired subclones (lanes 2 and 3, 5 and 6, and 8 and 9). The sizes of the PCR amplicons specific for two (Hp51, AM292592), three (Hp5, AM292581), four (Hp63, AM292557), and five (alcag202, AM279268) EPIYA motifs are depicted in lane 10. (B) Aligned nucleotide sequences of the EPIYA PCR amplicons for the paired subclones. (C) Aligned deduced peptide sequences of CagA protein with different numbers of EPIYA-C repeats in the paired subclones. (D) Functional expression of CagA protein from the paired subclones, following in vitro infection of adenocarcinoma cell line AGS. Note the presence of double bands in the mixed clinical isolates (lanes 1, 4, and 7) and the corresponding single bands in the individual subclones (lanes 2 and 3, 5 and 6, and 8 and 9). The GenBank/EMBL/DDBJ accession numbers are as follows: Hp77-2, AM292594; Hp77-5, AM292593; alcag4-1, AM292554; alcag4-4, AM292555; alcag40-2, AM292577; and alcag40-4, AM292578.

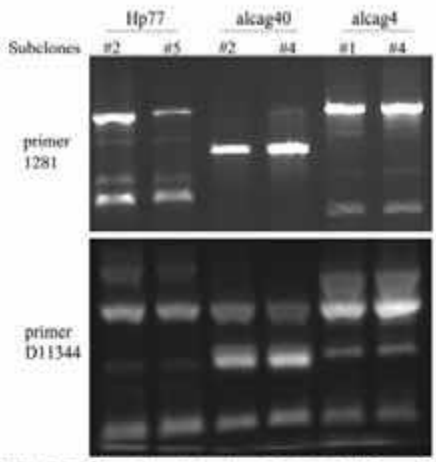

FIG. 3. RAPD profiles of closely related paired subclones Hp77-2-Hp77-5, alcag4-2-alcag4-4, and alcag40-2-alcag40-4 isolated from the same respective patients. Primers 1281 and D11344 were used as described previously (2). The patterns indicate high relatedness among the paired subclones.

TABLE 1. Comparison between PCR methods for the correct determination of the number of EPIYA motifs within CagA in single clone isolates

No. of EPIYA motifs*	No. of strains tested	No. of correct predictions	
		This study	Argent et al. (7)
0	8	6	2
2	1	1	0
3	9	9	9
4	13	13	12
5	3	3	2

* As determined by sequencing.

our method was 100% accurate in all cases of identification of *cagA*-negative strains, as well as predicting four or five EPIYA repeats (Table 1). In addition, when we compared the two techniques in five cases of *H. pylori* isolates containing two subclones with different number of EPIYA repeats, isolated from the same patient, our method accurately identified the presence of these subclones and furthermore predicted the right number of EPIYA repeats in all cases (Table 2). These results suggest that our prediction method based upon the size of PCR amplicons is quite robust since it can give accurate predictions, especially in the presence of multiple infective subclones expressing CagA protein with different numbers of EPIYA motifs.

Differences in the number of EPIYA motifs within CagA protein in *H. pylori* isolates from children and adults. We classified the population of *cagA*-positive strains with regards to EPIYA number and type (Table 3). The overwhelming majority of our clinical strains were found to harbor the ABC combination of EPIYA motifs (adults [67.3%], children [72.1%]; Table 3). In 10 strains isolated from adults (17.2%) and 7 strains from children (16.3%) we determined the ABCC combination of EPIYA motifs, as a result of a 34-amino-acid

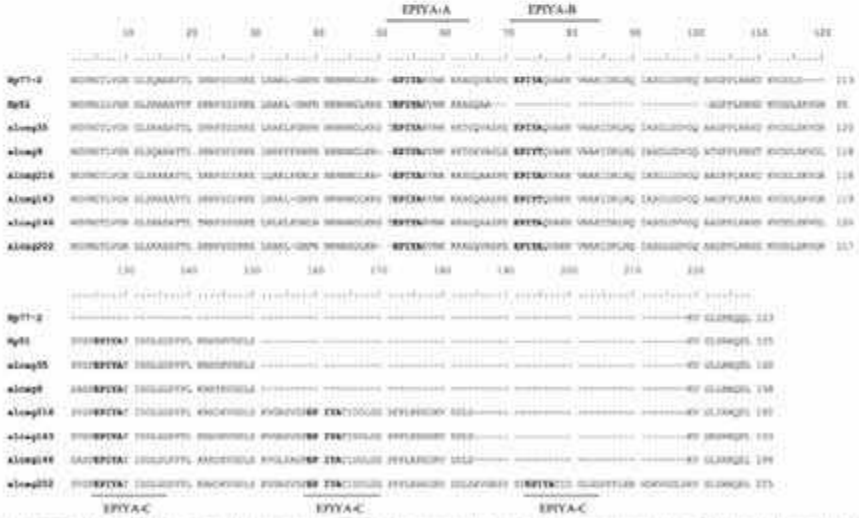

FIG. 4. Alignment of partial CagA peptide sequences derived from our analysis, depicting a representative collection of diverse CagA species with respect to the number and types of EPIYA motifs. The GenBank/EMBL/DDBJ accession numbers are as follows: Hp77-2, AM292594; Hp51, AM292592; alcag35, AM279305; alcag9, AM279304; alcag216, AM279293; alcag163, AM279292; alcag146, AM279291; and alcag202, AM279288.

TABLE 2. Determination of EPIYA motifs within CagA in mixed isolates containing multiple closely related subclones isolated from the same host

Case	Accession no.[a]	EPIYA status	
		This study	Argent et al. (7)
alcag7	AM295790-1	ABC-ABCC	ABCC
alcag80	AM295577-8	ABC-ABCC	ABCC
alcag4	AM292554-5	ABC-ABCCC	ABCCC
Hp77	AM292593-4	AB-ABC	No pattern
cva9	AM292596-7	AB-ABC	ABC

[a] That is, the GenBank/EMBL/DDBJ accession numbers of individual paired subclones.

TABLE 3. CagA diversity with regard to the number and type of EPIYA motifs in *cagA*-positive clinical isolates

Strain group and no. of EPIYA motifs	Type(s) of EPIYA motif	Adults ($n = 58$)[a]		Children ($n = 43$)[a]	
		n	%	*n*	%
Single strains					
2	AC	0	0	1	2.3
3	ABC	39	67.3	31	72.1
4	ABCC	10	17.2	7	16.3
5	ABCCC	2	3.5	0	0
5	ABABC	1	1.7	0	0
Mixed isolates					
2 and 3	AB-ABC	1	1.7	1	2.3
3 and 4	ABC-ABCC	3	5.2	3	7.0
3 and 5	ABC-ABCCC	1	1.7	0	0
4 and 6	ABCC-ABCCCC	1	1.7	0	0

[a] Percentages refer to the total number of *cagA*-positive isolates within each population. *n*, number of isolates.

repetition including the EPIYA-C (Fig. 4). The absence of the EPIYA-B motif was detected in only one isolate derived from children (Hp51, AM292592). Finally, exclusively within adults, two strains were found to harbor five EPIYA motifs in the ABCCC combination and one strain the ABABC combination (Table 3 and Fig. 4). No other combinations of TPM-A, -B, or -C were observed within our representative sample population. With regard to the multiple infections, six cases (three adult and three children) involved subclones expressing CagA protein with three and four EPIYA motifs in the ABC-ABCC combination and two cases with AB-ABC combination (Table 3). Interestingly, as observed in the case of single-clone isolates, subclones derived from multiple infections, expressing CagA protein with five or more EPIYA motifs, were detected only within adults. These data suggest that although no qualitative or quantitative difference exists between the strains isolated from adults and children, with regard to three or four EPIYA motifs, strains expressing CagA protein with five or more EPIYA motifs were detected exclusively within the adult population.

DISCUSSION

In the present study, we proposed a simple strategy by which accurate prediction of the *cagA* status, as well as the number and type of EPIYA motifs, involves just one single-step PCR amplification of the region encoding for the EPIYA motifs in the *cagA* gene. After *H. pylori* isolation from the gastric biopsy and DNA extraction, samples were subjected to EPIYA PCR. We showed that the absence of EPIYA PCR amplicon can accurately identify *cagA*-negative cases and therefore no prior characterization of the *cagA* status of the isolates is needed. In the event of a PCR amplicon with sizes of 470 or 570 bp we could safely assume the presence of an ABC or an ABCC combination of EPIYA motifs within CagA. We verified the accuracy of such predictions concerning three or four EPIYA motifs by sequencing in 93 cases of the 101 *cagA*-positive isolates analyzed within our study. More specifically, 70 of 101 *cagA*-positive strains carried three EPIYA motifs arranged in the ABC combination, 17 strains harbored four EPIYA motifs in the ABCC combination, and six cases involved mixed infections of the ABC-ABCC type. Therefore, in the overwhelming majority of cases (92%), the size of the PCR amplicon can safely predict the number and type of EPIYA motifs present. However, in the case of EPIYA PCR amplicons at 370 or 670 bp (8% of the cases) corresponding to CagA species with two EPIYA repeats (AB or AC combination) or more than four EPIYA repeats (ABCCC or ABABC combination), sequencing was required to establish the exact type of EPIYA motif combination, although prediction of the correct number of EPIYA motifs was possible. Moreover, our approach enabled us to accurately predict the number of EPIYA motifs in cases of mixed infections, where closely related subclones bearing the same RAPD profile, but divergent numbers of EPIYA motifs, were isolated from the same host. It is a common observation that such a pool of *H. pylori* clones may exist in a dynamic equilibrium within potentially all *H. pylori*-positive hosts (11) and reflect the continuous selective environmental pressure created by acidity and individual host immune responses, as well as factors relating to antibiotic consumption and diet (10, 31). Such *H. pylori* isolates may contain two or more closely related subclones bearing the same RAPD profile and therefore be indistinguishable in common clinical practice. This is particularly important, since these divergent CagA species were shown to be normally expressed (5; the present study) and can induce various degrees of humminghird phenotype upon infection of gastric epithelial cells (5). Therefore, by applying our strategy, we could detect the presence of such subclones with divergent EPIYA motifs and isolate them. However, it does not discriminate between coinfecting strains with different genotypes harboring CagA with the same number of EPIYA motifs.

Our approach is especially suited to clinical microbiology laboratories where *H. pylori* isolation from gastric biopsies is often made by the sweeping method and therefore the presence of multiple closely related subclones within an *H. pylori* isolate is possible. On the contrary, the presence of such subclones may severely weaken the specificity of the previously proposed PCR approach for the determination of the EPIYA motifs using primers specific to the EPIYA-A, -B, or -C coding sequences (7). Indeed, in our hands, such a technique was accurate only in the case of single-clone isolates, and it proved less powerful in cases of mixed infections. Finally, our proposed scheme involves only a single-step PCR and therefore is more cost-effective than the already published method which utilizes three different primer sets.

Based upon the positivity rates of our PCR amplification assay, we determined *cagA* presence in 75% of the adult and children populations analyzed. Our data are in line with CagA seroprevalence in Greece, which has been reported at 77.4% and was found to be constant across gender and age (4). More than 92% of the *cagA*-positive strains harbored a CagA protein with three or four EPIYA motifs arranged in the ABC or ABCC combination, respectively. We found no Eastern type strains circulating within our population, although our primers were designed to also detect such cases. The presence of EPIYA-A and -B motifs was detected in all our *cagA*-positive isolates, with the exception of only one strain harboring an AC EPIYA motif. Furthermore, we observed no differences between adults and children with reference to the distribution of strains expressing CagA with one or two EPIYA-C repeats. It was, however, exclusively within the adult population that we detected the presence of strains with more than five EPIYA motifs (5 isolates out of 135 strains analyzed). A much higher number of cases should be analyzed overall in order to perform robust statistical analysis and compare the prevalence of such strains in adults and children. However, our findings may suggest that *cagA*-positive strains can acquire additional EPIYA-C motifs throughout adulthood and thus contribute to *H. pylori* pathogenesis at a later age. *H. pylori* have been shown to exhibit unique genetic variability through out chronic *H. pylori* infection, and these genetic changes may be directed by homologous recombination (19).

We have developed a rapid approach for the accurate characterization of EPIYA motifs within the carboxyl terminus of CagA protein, even in the presence of multiple infecting subclones and successfully typed *H. pylori* strains from Greek adults and children. We are applying our approach to the study of the potential association of CagA divergence within the carboxyl-terminal end and the clinical outcome of the *H. pylori*-associated disease. Further analysis of our strains with respect to their ability to successfully translocate the CagA protein and interact with intracellular effectors within the gastric epithelial cells, through phosphorylation-dependent and -independent interactions, may provide more clues regarding their true pathogenic potential.

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Helicobacter pylori Infection in Symptomatic HIV-Seropositive and -Seronegative Patients: A Case–Control Study

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ABSTRACT

We conducted a case–control study in a Greek hospital to evaluate the prevalence and morbidity of *Helicobacter pylori* in HIV-infected patients. HIV-seropositive patients were infected by *H. pylori* less often than HIV-seronegative controls [12/58 (20.7%) versus 38/58 (65.5%), $p < 0.001$]. The mean CD4 count was lower for *H. pylori*-negative than *H. pylori*-positive HIV-infected patients ($p < 0.007$). Also, among HIV patients, prior use of antibiotics or proton pump inhibitors was more common in those without *H. pylori* infection, however, this difference was not statistically significant ($p = 0.06$). The grading of the density of *H. pylori* infection and the grading of the histomorphological findings according to the Sydney classification were similar between HIV-seropositive and -seronegative patients with *H. pylori* infection.

INTRODUCTION

PUBLISHED DATA DURING THE PAST DECADE indicate a low prevalence of *Helicobacter pylori* in HIV-infected persons, especially in those with overt acquired immunodeficiency syndrome (AIDS) or those with a lower CD4 count.[1–3] The increased use of antibiotics in HIV patients[3] and changes in immune response that accompany the decline of the CD4 cells[4,5] are some of the factors that have been proposed to play a role in the low prevalence of *H. pylori* in patients with HIV. Apart from the factors that may be related to lower *H. pylori* prevalence in patients with HIV infection, confusion also exists regarding the virulence and pathogenicity of the bacterium in this patient population.

Thus, we conducted a case–control study in a Greek hospital offering gastrointestinal (GI) endoscopy services to inpatients and outpatients, including HIV-infected patients, in order to evaluate the prevalence of *H. pylori* in HIV patients, compared to HIV noninfected controls.

MATERIALS AND METHODS

The study population included patients who visited the GI Endoscopy Department of the First IKA Hospital, a 250-bed tertiary hospital in Athens, Greece, during a period of 11.5 years (April 1994–September 2005). Data were retrospectively collected for all HIV-infected patients who were referred to the Department for upper GI endoscopy. For each HIV-infected patient, the next patient not infected with HIV who underwent upper GI endoscopy at the same Department during the same day served as control. We used two methods for the detection of *H. pylori*, the rapid urease CLO test (Ballard Medical Products, Draper, UT), which has a good sensitivity (90%) and specificity (100%), and histology (Giemsa stain). Histological specimens were taken from the body and the antrum of the stomach.

The main outcome that we examined was the prevalence of *H. pylori* in HIV-infected patients and controls. The results in HIV-seropositive patients were further analyzed with respect to the CD4 count. In addition, the association between exposure

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to antibiotics active against *H. pylori*, or proton pump inhibitors (PPIs), administered during the year and month, respectively, prior to the date of the upper GI endoscopy and diagnosis of *H. pylori* infection was evaluated. The upper GI endoscopic and histological findings in HIV-seropositive and HIV-seronegative patients with and without *H. pylori* were compared. To categorize these findings we used the Sydney classification for gastritis plus data regarding the presence of peptic ulcer.[6,7]

Finally, we also analyzed the data according to the etiology of histologically confirmed gastritis. For this purpose our histological specimens were also examined for the presence of opportunistic infections. The presence of cytomegalovirus (CMV) and herpes simplex virus (HSV) was confirmed by *in situ* hybridization, whereas *Mycobacteria* were detected with the *Ziehl–Neelsen stain* and *Cryptosporidium* or other parasites by microscopy.

For the analysis of our results we used the statistical program SPSS 10 (SPSS Inc., Chicago, IL). Categorical variables were compared by chi-square and Fischer exact tests. For continuous variables we used the Student's *t* test or the Mann–Whitney test for normally and nonnormally distributed variables, respectively. A p value of <0.05 denoted statistical significance.

RESULTS

We enrolled 58 patients with HIV infection aged 22–67 years old (mean age 40.5 years), of whom 47 were males. The control group was 58 HIV-seronegative patients aged 16–79 years old (mean age 46.3 years), of whom 31 were males. Fewer patients in the HIV-seropositive group compared to the control group of HIV-seronegative patients had *H. pylori* infection, both according to histology and CLO test [12/58 (20.7%) versus 38/58 (65.5%) and 12/58 (20.7%) versus 36/58 (62%), respectively; in both comparisons $p < 0.001$] (Table 1).

Data regarding the number of CD4 cells were available for 34 HIV-seropositive patients. Among these patients, the mean value of CD4 cells was lower for *H. pylori* negative (*Hp*−) than *H. pylori* positive (*Hp*+) patients (mean ± standard deviation, 187.2 cells/μl ± 274.8 versus 316.2 cells/μl ± 362.2, $p < 0.007$). However, when HIV-seropositive patients were grouped by using the CD4 cutoff of 200 cells/μl, the difference was not found to be statistically significant; 2/21 (9.5%) patients with less and 3/13 (23%) patients with more than 200 CD4 cells/μl were *Hp*+ ($p = 0.35$).

The prevalence of *H. pylori* in HIV-seropositive patients was further evaluated in relation to the prior use of antibiotics effective against *H. pylori* and PPIs in 27 patients for whom these data were available. There was no statistically significant difference in the prevalence of *H. pylori* infection between patients who received antibiotics active against *H. pylori* (with or without PPIs) and patients who did not receive such medications [1/13 (7.7%) versus 5/14 (35.7%), $p = 0.16$]. An almost statistically significant difference was noted between exposure to antibiotics active against *H. pylori* or PPIs and absence of *H. pylori* infection [1/15 (6.7%) versus 5/12 patients (41.7%), $p = 0.06$].

The findings of upper endoscopy and histology according to the Sydney classification are presented in Table 2, for both HIV-seropositive and -seronegative patients. Endoscopic evidence of gastritis in patients infected with *H. pylori* was less common among HIV-seropositive than -seronegative patients [5/12 (41.5%) versus 35/38 (92%), $p = 0.001$]; however, no difference was found between the two compared groups regarding the presence of peptic ulcer [1/12 (8%) versus 11/38 (29%), $p = 0.25$] (Table 2). Furthermore, both the grading of the density of *H. pylori* (Table 1) and the grading of the histomorphological findings (Table 2) were similar between HIV-seropositive and -seronegative patients with *H. pylori*. Finally, as shown in Table 3, gastritis due to infections other than *H. pylori* occurred only in HIV-seropositive patients.

DISCUSSION

The main finding of our study is that *H. pylori* infection was less likely in HIV-seropositive than HIV-seronegative patients in our study population. In addition, among the studied HIV-seropositive patients, the mean CD4 count was higher in those with *H. pylori* infection compared to those without this infection. Also, among HIV-seropositive patients, although a non-statistically significant result, exposure to antibiotics with activity against *H. pylori* or PPIs was less likely in those with *H. pylori* infection. Finally, both the grading of the density of *H. pylori* infection and the grading of the histomorphological find-

TABLE 1. RESULTS OF THE UREASE TEST AND HISTOLOGY FOR THE IDENTIFICATION OF *H. pylori* (Hp) IN THE GROUPS OF HIV-SEROPOSITIVE (+) AND -SERONEGATIVE (−) PATIENTS

	HIV (+) *Hp* (+) ($n = 12$)	*HIV* (+) *Hp* (−) ($n = 46$)	*HIV* (−) *Hp* (+) ($n = 38$)	*HIV* (−) *Hp* (−) ($n = 20$)
Hp density on histology				
Normal	0	46/46 (100%)	0	20/20 (100%)
Mild	2/12 (16.6%)	0	5/38 (13.1%)	0
Moderate	8/12 (66.8%)	0	21/38 (55.3%)	0
Marked	2/12 (16.6%)	0	12/38 (31.6%)	0
CLO test				
Negative	0	46 (100%)	2/38 (5.3%)	20/20 (100%)
Positive	12 (100%)	0	36/38 (94.7%)	0

TABLE 2. FINDINGS FROM UPPER ENDOSCOPY AND HISTOLOGY IN THE GROUPS OF HIV AND *H. pylori* (Hp) POSITIVE (+) OR NEGATIVE (−) PATIENTS

	HIV (+) *Hp* (+) (*n* = 12)	*HIV* (+) *Hp* (−) (*n* = 46)	*HIV* (−) *Hp* (+) (*n* = 38)	*HIV* (−) *Hp* (−) (*n* = 20)
Endoscopic findings				
Normal	7/12 (58.5%)	14/46 (30.5%)	3/38 (8%)	5/20 (25%)
Gastritis	5/12 (41.5%)	32/46 (69.5%)	35/38 (92%)	15/20 (75%)
Erythematous/exudative	4	18	27	8
Flat erosive	0	9	5	5
Raised erosive	1	1	3	0
Atrophic	0	1	0	0
Hemorrhagic	0	1	0	1
Bile	0	2	0	1
Peptic ulcer[a]	1/12 (8%)	4/46 (8.7%)	11/38 (29%)	1/20 (5%)
Gastric	0	2	3	1
Duodenal	1	2	8	0
Classification of gastritis according to localization				
Pan-gastritis	2/5 (40%)	17/32 (53.1%)	17/35 (48.5%)	9/15 (60%)
Limited to the body	1/5 (20%)	5/32 (15.6%)	3/35 (8.5%)	0
Limited to the antrum	2/5 (40%)	10/32 (31.3%)	15/35 (43%)	6/15 (40%)
Histomorphological findings[b]				
Normal	0	24/46 (52.2%)	0	9/20 (45%)
Mononuclear cells	6/12 (50%)	7/46 (15.2%)	16/38 (42.1%)	6/29 (30%)
Neutrophils	6/12 (50%)	3/46 (6.5%)	21/38 (55.3%)	1/20 (5%)
Atrophy	0	1/46 (2.1%)	0	0
Intestinal metaplasia	0	2/46 (4.4%)	1/38 (2.6%)	0
Edematous, congestive, hemorrhagic wall without other findings	0	9/46 (19.6%)	0	4/20 (20%)

[a]Patients with peptic ulcer also had some form of gastritis.
[b]Presence or absence of a histomorphological feature.

ings were similar between HIV-seropositive and -seronegative patients with *H. pylori*, indicating similarities between cases and controls once infection with *H. pylori* was present.

The results of our study provide further support to previous investigations, most of which showed that the prevalence of *H. pylori* in adults with HIV infection is lower than in the general population,[1,8] whereas this may not be true for the pediatric HIV-seropositive population.[9] In addition, patients with a more advanced HIV infection are less likely to be *Hp*+.[3,4] The results of experimental and clinical studies indicate that the presence of CD4 is crucial for the development of *H. pylori*-associated gastritis.[10–12] Compared to matched controls, it is likely

TABLE 3. CLASSIFICATION OF HISTOLOGICALLY CONFIRMED GASTRITIS ACCORDING TO ETIOLOGY IN THE HIV (+) AND HIV (−) GROUPS OF PATIENTS AND CONTROLS[a]

Etiology	*HIV* (+) *Hp* (+) (*n* = 12)	*HIV* (+) *Hp* (−) (*n* = 46)	*HIV* (−) *Hp* (+) (*n* = 38)	*HIV* (−) *Hp* (−) (*n* = 20)
Nonidentified etiology	0	5/22 (22.8%)	0	2/11 (18.2%)
Infectious	12/12 (100%)	2/22 (9%)	38/38 (100%)	0
Hp nonatrophic (type B)	12	0	38	0
Cytomegalovirus	0	1	0	0
Cryptosporidium	0	1	0	0
Chemotaxic agents (type C)	0	11/22 (50%)	0	7/11 (63.6%)
NSAID	0	7	0	5
Alcohol	0	2	0	1
Bile	0	2	0	1
Distinct forms	0	4/22 (18.2%)	0	2/11 (18.2%)
Eosinophilic	0	1	0	1
Portal gastropathy	0	3	0	1

[a]There were no cases of autoimmune gastritis, *Hp* atrophic gastritis, gastritis due to herpes simplex virus, mycobacteria, parasites, chemotaxic agents (other than those mentioned in the table), neoplasms, lymphoma, or other distinct forms.

that patients with HIV infection receive more antibiotics due to the need for primary or secondary prophylaxis for opportunistic infections.

Our study is not without limitations. First, we had data regarding exposure to antibiotics and CD4 count only for a subset of patients. Also, someone may suggest that another control group may have been more appropriate, for example, HIV-seronegative patients matched to cases for age, gender, and important factors associated with *H. pylori* infection such as socioeconomic status and family size. However, the matching method we used accounts for possible changes in the prevalence of peptic ulcer in seropositive and seronegative HIV patients during our study. Finally, there was a difference in the percentage of male patients between cases and controls, but this is mainly related to the high percentage of male homosexuals among HIV-infected patients in Greece. Nevertheless, published studies do not report a true difference in *H. pylori* prevalence between males and females.

In conclusion, we found that HIV-seropositive patients were infected with *H. pylori* less often than the general population. Among patients with HIV, those with decreased CD4 cells were less likely to be coinfected with this bacterium.

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In Vitro and In Vivo Activities of Chios Mastic Gum Extracts and Constituents against *Helicobacter pylori*▽

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The extracts and pure major constituents of Chios mastic gum (resin of *Pistacia lentiscus* var. *chia*) were tested for their activities against *Helicobacter pylori*. A total mastic extract without polymer (TMEWP) was prepared after removal of the contained insoluble polymer in order to ameliorate solubility and enhance in vivo activity. Administration of TMEWP to *H. pylori* SS1-infected mice over the period of 3 months with an average dose of 0.75 mg/day led to an approximately 30-fold reduction in the *H. pylori* colonization (1.5 log CFU/g of tissue). However, no attenuation in the *H. pylori*-associated chronic inflammatory infiltration and the activity of chronic gastritis was observed. To further characterize potential active mastic constituents, the TMEWP was separated into an acidic and a neutral fraction. Both were extensively characterized by nuclear magnetic resonance and mass spectroscopy to elucidate the structure of the components contained within each fraction. After chromatographic separation, the acid fraction gave the major triterpenic acids, while the neutral fraction gave several triterpenic alcohols and aldehydes. Mastic extracts and isolated pure triterpenic acids were tested for in vitro activity against a panel of 11 *H. pylori* clinical strains. The acid fraction was found to be the most active extract (minimum bactericidal concentration [MBC], 0.139 mg/ml), and the most active pure compound was isomasticadienolic acid (MBC, 0.202 mg/ml [0.443 mM]). Our results show that administration of TMEWP may be effective in reducing *H. pylori* colonization and that the major triterpenic acids in the acid extract may be responsible for such an activity.

Pistacia lentiscus L. is an evergreen shrub of the *Anacardiaceae* family, very common in the eastern Mediterranean area. The variety chia (Duham) is uniquely cultivated in southern Chios, a Greek island in the Aegean. The resin of that plant, mastic gum, is obtained as an exudate after "hurting" the trunk and branches.

Mastic gum has been used in traditional Greek medicine for various gastrointestinal disorders like gastralgia, dyspepsia, and peptic ulcer for more than 2,500 years. Ancient Greek physicians, such as Hippocrates, Dioscorides, Theophrastos, and Galenos, mentioned its properties and recommended its use. In modern times, it is 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 gastralgia and protection against peptic ulcer. Early studies involving mastic administration to rats with experimentally induced gastric and duodenal ulcers indicated a significant decrease of free acidity (2). Moreover, a double-blind clinical trial carried out on patients with symptomatic and endoscopically proven duodenal ulcer showed increased symptomatic relief in patients on mastic (1 g daily) compared to patients on placebo, while endoscopically proven healing occurred in 70% of the patients on mastic (1).

In 1983, Barry Marshall and Robin Warren suggested that gastric inflammation and peptic ulceration were the result of an infection caused by *Helicobacter pylori* (34). In the years to follow, the presence of *H. pylori* infection was shown to be the etiologic determinant of chronic active gastritis and a major risk factor for the development of peptic ulcer disease, gastric atrophy, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (23). Antibiotic eradication schemes have proved very effective in clearing the infection; however, low patient compliance and the development of antibiotic resistance have created the need for new *H. pylori* eradication strategies. Mastic gum has been reported to possess considerable in vitro antibacterial and antifungal activities (32). A few years ago, it was specifically reported to be effective against *Helicobacter pylori* in vitro (7, 12, 21). However, in a more recent in vivo study of *H. pylori* infection, the activity of mastic gum was compared with antibiotic eradication schemes, and after a 7-day therapy no eradication of the bacterium from the stomachs of mice receiving mastic was observed (17). Finally, *H. pylori*-positive patients were treated with mastic capsules for 7 days, and they all remained *H. pylori* positive after the administration (6). The last two studies concluded that no "antibiotic-like" activity should be expected from crude mastic.

The crude resin that was used in all previous studies contained a high percentage (30%) of an insoluble and sticky polymer (poly-β-myrcene) (33) that obviously hinders its oral administration and reduces the bioavailability of the contained active compounds. To bypass such problems, we prepared a

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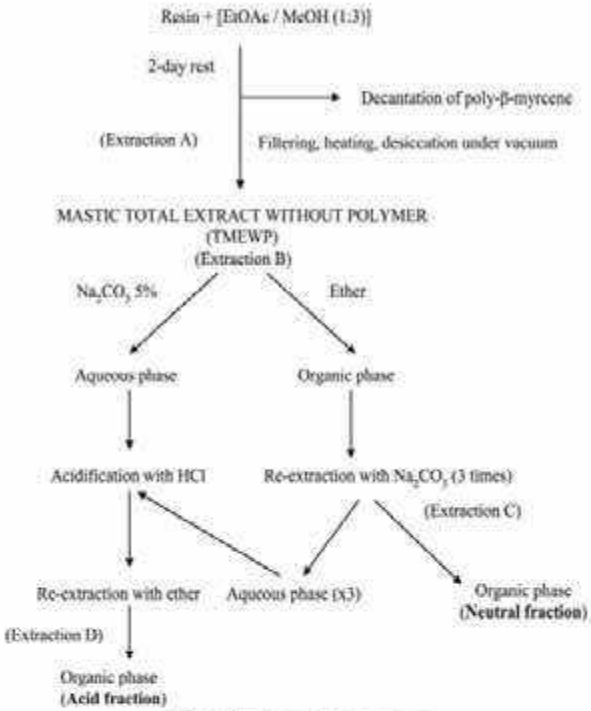

FIG. 1. Extraction of Chios mastic gum.

total mastic extract without polymer (TMEWP) and tested its activity against *H. pylori* in mice infected with the *H. pylori* SS1 strain. In addition, well-characterized mastic gum fractions as well as isolated pure compounds were tested in vitro against *H. pylori* in an effort to identify the most active constituents.

MATERIALS AND METHODS

General procedures. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker DRX 400 and Bruker AC 200 spectrometers (^{1}H [400 and 200 MHz] and ^{13}C [50 MHz]; chemical shifts are expressed in ppm downfield from tetramethylsilane. The ^{1}H-^{1}H and the ^{1}H-^{13}C NMR experiments (distortionless enhancement by polarization transfer, correlated spectroscopy [COSY], COSY for long-range couplings, heteronuclear multiple quantum coherence, heteronuclear multiple-bond correlation, and nuclear Overhauser effect spectroscopy [NOESY]) were performed using standard Bruker microprograms. Gas chromatography-mass spectroscopy (MS) analysis was performed on a Finnigan GCQ Plus mass spectrometer. Electron impact and chemical ionization-MS spectra were determined on a Finnigan GCQ Plus mass spectrometer using CH_4 as the chemical ionization reagent and high-resolution mass spectrometry on an Akl-MS-90 spectrometer. Medium-pressure liquid chromatography (MPLC) was performed with a Büchi model 688 apparatus on columns containing Si gel (type 60; 20 to 40 μm; Merck).

Extraction process. Commercial mastic gum was supplied by the Chios Mastic Growers Association, which is the exclusive worldwide producer of the resin. A quantity of mastic gum (500 g) was diluted in ethyl acetate (500 ml), and then methanol (1,500 ml) was added (Fig. 1). The mixture was let stand, and after a period of 2 days, a layer of poly-β-myrcene (150 g) was decanted. The clear supernatant solution was obtained by filtration, and the solvent mixture was evaporated in a rotary evaporator at 45°C with an 80-kPa vacuum (extraction A). The resulting semisolid residue was dried in a desiccator at 70°C and 1,000 mbar vacuum and gave a white powder (350 g). The TMEWP was freely soluble in ethanol, which is feasible for the crude resin only under protracted heating. TMEWP was partitioned between aqueous 5% Na_2CO_3 (1 liter) and ether (3.5 liter) as described by Barton (5) (extraction B). The organic phase was reextracted three times with 5% Na_2CO_3 (1 liter each time) (extraction C) and afforded the neutral fraction of mastic (135 g) as the organic phase. The aqueous phase was added to that of extraction B and acidified with 1 N HCl (3 liters). The acidic solution was reextracted with ether (6 liters) (extraction D), and the organic phase afforded the acid fraction of mastic (190 g).

Comparison of crude mastic gum and TMEWP chemical profile. Crude mastic gum and the TMEWP were submitted to thin-layer chromatography over silica gel using a mixture of CH_2Cl_2 and methanol (MeOH) (98:2) as the solvent system. The chromatographic zones were detected after spraying with a solution of vanillin and sulfuric acid in methanol. The chromatograms of both substances were identical except for poly-β-myrcene, which was present only in the crude resin (data not shown).

Isolation of pure triterpenic acids and neutral compounds. A part of the acidic fraction (20 g) was submitted to MPLC over normal-phase silica gel first with a cyclohexane-CH_2Cl_2 gradient (from cyclohexane-CH_2Cl_2 at 50:50 to CH_2Cl_2 at 100%) and then with a CH_2Cl_2-MeOH gradient (from CH_2Cl_2 at 100% to CH_2Cl_2-MeOH at 80:20). The total solvent volume used was 17.3 liters. A total of 24 fractions were obtained, the separation of which was decided on the basis of thin-layer chromatography (with several systems used as solvents), and the chromatographic zones were detected as mentioned above (the same separation procedure was followed in all chromatographic separations mentioned below). Fraction 11 (1.252 g) was separated by MPLC over normal-phase silica gel with a CH_2Cl_2-MeOH gradient (from CH_2Cl_2 at 100% to CH_2Cl_2-MeOH at 80:20).

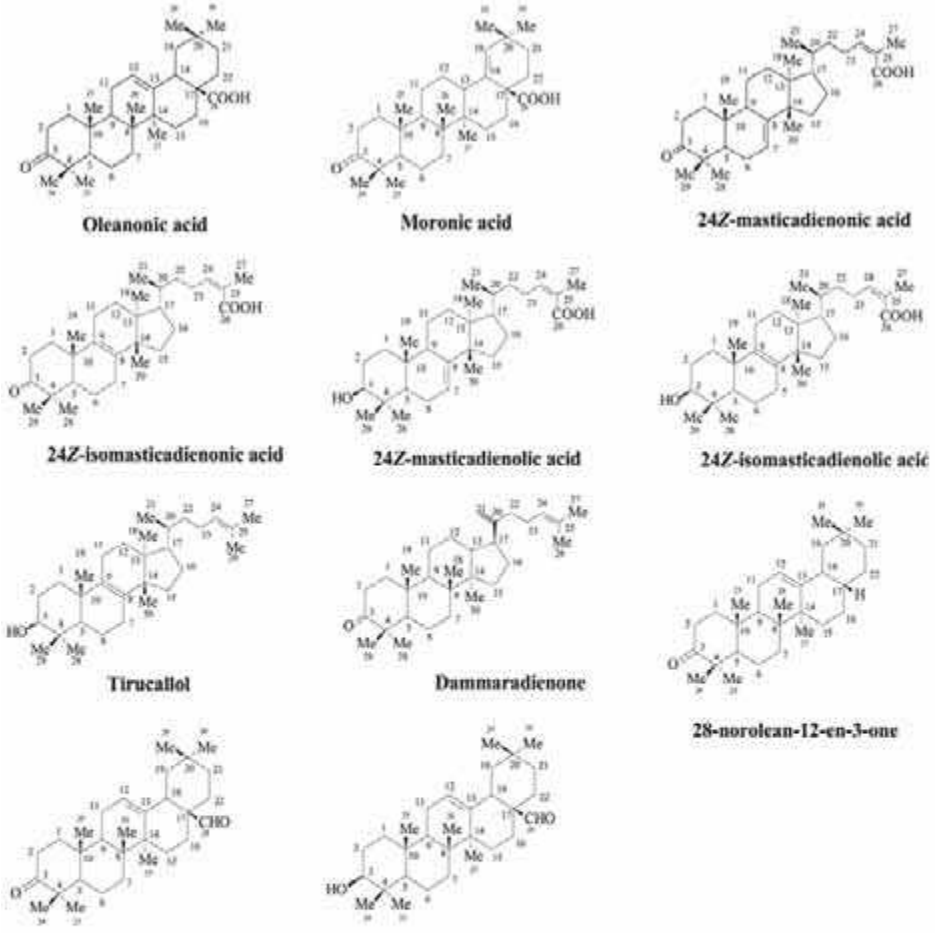

FIG. 2. Triterpenic compounds isolated from mastic acidic and neutral fractions.

total solvent volume used, 2.1 liters) and afforded oleanonic acid (515 mg) (29, 30) and moronic acid (338 mg) (13). Fraction 12 (2.431 g) was separated by MPLC over normal-phase silica gel eluted with a CH_2Cl_2-MeOH gradient (from CH_2Cl_2 at 100% to CH_2Cl_2-MeOH at 98/20; total solvent volume used, 3.3 liters) and afforded 24*Z*-masticadienonic acid (1.1 g) (5, 8, 22) and 24*Z*-isomasticadienonic acid (1.0 g) (26). Fraction 17 (198 mg) was separated by column chromatography over silica gel eluted with a CH_2Cl_2-MeOH gradient (from CH_2Cl_2-MeOH from 99/1 to 80/20; total solvent volume used, 1.1 liter) and afforded 24*Z*-masticadienolic acid (85 mg) (9, 22) and 24*Z*-isomasticadienolic acid (92 mg) (24). The molecular structures are displayed in Fig. 2. All the above constituents were identified by one-dimensional (1D) and 2D NMR and MS and by comparison with data in the literature. Full NMR data for 24*Z*-isomasticadienonic acid, which have never been reported, are presented below.

24*Z*-Isomasticadienonic acid. White "powder," m.p. 166 to 167°C; $[\alpha]_D$ +34° (*c 1.10*, MeOH); EI-MS (*m/z*): 454 (34), 439 (100), 421 (62), 393 (13), 271 (11), 257 (19). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 0.70 (3H, s, H-18), 0.82 (3H, s, H-30), 0.86 (3H, d, J = 5.8 Hz, H-21), 0.98 (3H, s, H-28), 1.02 (3H, s, H-19), 1.04 (3H, s, H-29), 1.08 (1H, H-22a), 1.19 (1H, H-15a), 1.38 (1H, H-20), 1.45 (1H, H-17), 1.46 (1H, H-22β), 1.48 (1H, H-15β), 1.57 (1H, H-1a), 1.64 (1H, H-5), 1.67 (2H, H-12), 1.80 (2H, H-6), 1.84 (3H, s, H-27), 1.88 (1H, H-16a), 1.90 (2H, H-11), 1.99 (1H, H-16β), 1.93 (1H, H-1β), 2.06 (2H, dd, J = 7 Hz, 14 Hz, H-7), 2.39 (1H, H-23a), 2.42 (1H, H-2a), 2.49 (1H, H-2β), 2.51 (1H, H-23b), 6.01 (1H, t, J = 7 Hz, H-24). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ = 15.51 (C-18), 18.53 (C-21), 19.74 (C-27), 20.22 (C-6), 20.51 (C-19), 21.09 (C-28), 21.32 (C-16), 24.15 (C-30), 26.65 (C-29), 26.83 (C-23), 27.42 (C-7), 28.01 (C-11), 29.74 (C-15), 30.65 (C-12), 34.55 (C-2), 35.51 (C-1), 35.80 (C-22), 36.39 (C-20), 37.09 (C-10), 49.59 (C-13), 47.23 (C-4), 48.99 (C-14), 49.99 (C-17), 51.42 (C-5), 125.81 (C-25), 132.61 (C-9), 134.63 (C-8), 147.35 (C-24), 173.40 (C-26), 218.32 (C-3).

A part of the neutral fraction (17.2 g) was submitted to column liquid chromatography over normal-phase silica gel with a cyclohexane-CH_2Cl_2 gradient

(from cyclohexane at 100% to CH_2Cl_2 at 100%) to afford 22 fractions. The total solvent volume used was 21 liters. Fraction 5 (839 mg) was separated by MPLC over normal-phase silica gel with a cyclohexane-CH_2Cl_2 gradient (from 90/10 to 100% CH_2Cl_2; total volume used, 2 liters) and afforded tirucallol (110 mg) (23) and dammaradienone (128 mg) (20). Fraction 8 (333 mg) was separated by MPLC over normal-phase silica gel eluted with a cyclohexane-CH_2Cl_2 gradient (from 80/20 to 100% CH_2Cl_2; total volume used, 1.5 liters) and afforded 28-norolean-12-en-3-one (206 mg) (20). Fraction 10 (306 mg) was separated by MPLC over normal-phase silica gel eluted with a cyclohexane-CH_2Cl_2 gradient (from 80/20 to 100% CH_2Cl_2; total volume used, 1.4 liters) and afforded oleanonic aldehyde (152 mg) (25) and oleanolic aldehyde (98 g) (2). The molecular structures are displayed in Fig. 2. All the above constituents were identified by 1D and 2D NMR and MS and by comparison with data in the published literature.

The neutral fraction was also submitted to analytical separation by gas chromatography-MS. Comparison of mass spectra with MS data library Wiley 225.l and data in the literature (4) resulted in the identification of the five compounds mentioned above as the major neutral ones, while several minor diterpenes or triterpenes with aldehyde, ketone, or hydroxyl groups were detected on the basis of molecular weight but not thoroughly identified.

In vitro test of mastic total extract, fractions, and pure compounds. Minimum bactericidal concentration (MBC) were evaluated utilizing *H. pylori* reference strain CCUG 36771 and another 16 clinical strains belonging to the Hellenic Pasteur Institute collection, isolated from gastric antrum biopsies from patients suffering from gastritis or duodenal or gastric ulcer (LAVHP-1 to LAVHP-10). *H. pylori* isolates were stored in brain heart infusion broth (BHIB) supplemented with 20% glycerol at −90°C. All strains prior to use were cultured twice under microaerophilic conditions (CampyPak Plus; Becton-Dickinson, Cockeysville, MD) for 24 h at 37°C, in Chalgren's-Wilkins (CHW) agar plates supplemented with 7% (vol/vol) horse blood and 1% (vol/vol) Vitox (Oxoid, Basingstoke, United Kingdom). Liquid cultures of *H. pylori* bacteria to a density of 10^9 CFU/ml were prepared by suspension in BHIB (Oxoid) supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, Scotland) and 0.25% yeast extract (Oxoid). Successive twofold dilutions of each mastic extract or pure compound in BHIB medium were placed in sterilized 96-well flat-bottom microplates (Xanetch, Numbrecht, Germany) within a total volume of 100 μl. All extracts were tested at a final concentration range of 0.049 to 1,560 mg/ml, with the exception of the acidic fraction, for which successive twofold dilutions ranged from 0.060 to 1,920 mg/ml. To each well containing the mastic dilutions, approximately 10^5 CFU of *H. pylori* bacteria were added within a 100-μl volume, and the microplates were incubated at 37°C for 24 h with continuous agitation under microaerophilic conditions. Thereafter, viability of *H. pylori* was evaluated by determination of viable CFU in CHW agar plates following incubation at 37°C for 48 h under microaerophilic conditions. The MBC was defined as the lowest concentration of mastic extract or pure compound that killed at least 99.9% of the CFU contained in the original inoculum. The mean MBC for each mastic preparation was determined as the average of three independent experiments.

Infection of mice with *H. pylori* strain SS1. Specific-pathogen-free 6- to 8-week-old female C57BL/6 mice were obtained from the Central Animal Facility of the Hellenic Pasteur Institute. They were housed according to relevant Greek national legislation, fed a commercial diet, and given water ad libitum, except as otherwise stated. *H. pylori* infections by the SS1 strain were carried out as described before (27, 28). Briefly, freshly prepared aliquots (100 μl; 10^9 CFU) of *H. pylori* strain SS1 in BHIB (Oxoid) were administered to mice via orogastric inoculation, three times within a week. Accordingly, all noninfected control mice were inoculated with the same volume of plain BHIB.

Administration of TMEWP in vivo. TMEWP was diluted into ethanol at a concentration of (80 mg/ml and then dissolved into a final aqueous solution of 180 μg/ml. The extract was administered through the animals' drinking water, starting 1 month following initial *H. pylori* infection and for 3 more months. The following groups of animals were included in the study: *H. pylori*-infected mice administered TMEWP (SMH; $n = 10$); uninfected mice administered TMEWP (SM; $n = 10$); and *H. pylori*-infected mice left untreated (SH; $n = 10$) as a control group. Animal weight was monitored throughout the whole observation period as a measure of health of the animals. Daily water consumption was measured, and a mean daily TMEWP consumption per animal was calculated to be 0.75 mg throughout the whole administration period. In addition to the above therapeutic in vivo protocol, we conducted a preliminary prophylactic study where C57BL/6 mice ($n = 5$) were administered TMEWP for a week prior to *H. pylori* infection, and we assessed *H. pylori* colonization 2 weeks after the initial infection.

Assessment of *H. pylori* colonization levels. At end of the 3-month observation period, blood samples were collected via the tail vein and animals were sacrificed by cervical dislocation. Excised stomachs were dissected along the lesser curvature, and *H. pylori* detection in the gastric tissue was accomplished by *H. pylori* quantitative culture and PCR. For *H. pylori* SS1 quantitative culturing, preweighed half-stomach samples were homogenized in thioglycolate medium (Oxoid), serially diluted in phosphate-buffered saline, and plated on CHW agar plates with 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; fluorocytosine, 5 μg/ml; all from Sigma, St. Louis, Mo.). The cultures were incubated under microaerophilic conditions at 37°C for up to 6 days. *H. pylori* colonies were visualized on the basis of urease activity, and results were expressed as log CFU per gram of gastric tissue. The minimum bacterial density detected by this method was 100 CFU per gram. Qualitative *H. pylori* detection in the gastric samples was performed by *H. pylori*-specific PCR utilizing primers for the *ureC* (*glmM*) gene as described before (18). Genomic DNA for the detection of *H. pylori* by PCR in tissue samples or bacterial colonies was isolated by using a DNeasy tissue kit (QIAGEN).

Histopathologic analysis of gastric tissue samples. Excised stomachs were opened along the lesser curvature, and the longitudinal half was fixed in 10% neutral buffered formalin solution, embedded in paraffin, and processed for histopathologic analysis. Antral, body, and cardioesophageal mucosa samples were examined in the same section. Eleven serial longitudinal 4-μm sections were cut from each specimen; 9 of them were stained with hematoxylin-eosin for evaluation of gastric inflammation, and 2 were stained by the May-Grünwald Giemsa stain method for the assessment of *H. pylori* colonization. The bacterial density and the pathological characteristics of the gastric mucosa were assessed according to the updated Sydney system (11). Histopathologic evaluation was performed with no prior knowledge of the identity of the samples by the histopathologist.

Determination of serum anti-*H. pylori* immunoglobulin G levels. Immunoglobulin G (IgG) levels of anti-*H. pylori* antibodies were detected in the serum samples collected by an in-house enzyme-linked immunosorbent assay method. Briefly, 15 μg of *H. pylori* SS1 antigen produced by sonication and subsequent dialysis (SpectraPor; cutoff pore size, 8 kDa) was used to coat 96-well plates. Collected mouse serum samples (diluted 1/50) were primarily incubated in the plates for 24 h at 4°C, and then rabbit anti-mouse IgG (whole molecule)-peroxidase conjugate (Sigma) was used for the secondary incubation (2 h at 37°C). Color was developed by addition of o-phenylenediamine (Sigma), and optical density at 492 nm was measured in a Sunrise microtiter plate reader (Tecan).

Statistical analysis. Analysis of the results from the in vivo experiments was performed with respect to *H. pylori* colonization by two-tailed unpaired *t* test with Welch correction and with respect to the associated gastritis by the Wilcoxon rank sum test due to the ordinal nature of the data. A P value of <0.05 was considered significant in both tests.

RESULTS

Isolation of TMEWP, acid and neutral fractions, and triterpenic compounds. The TMEWP was obtained from crude mastic gum in a 70% proportion using a modification of the method described by Barton (5). Ether was replaced by ethyl acetate, a more convenient and nonexplosive solvent, in order to minimize the risk when manipulating large quantities of resin.

The TMEWP was further divided into two fractions, an acidic and a neutral one. The acidic fraction of TMEWP after several chromatographic separations afforded the major triterpenic acids (Fig. 2) oleanonic acid (515 mg), moronic acid (338 mg), 24*Z*-masticadienonic acid (1.1 g), 24*Z*-isomasticadienonic acid (1.0 g), 24*Z*-masticadienolic acid (95 mg), and 24*Z*-isomasticadienolic acid (102 mg). The neutral fraction, after similar treatment, afforded five neutral triterpenic compounds: tirucallol (110 mg), dammaradienone (128 mg), 28-norolean-12-en-3-one (206 mg), oleanonic aldehyde (152 mg), and oleanolic aldehyde (98 mg).

All the above constituents were identified by NMR (^1H, ^{13}C, distortionless enhancement by polarization transfer, COSY, heteronuclear multiple quantum coherence, heteronuclear multiple-bond correlation, and NOESY) and MS and by com-

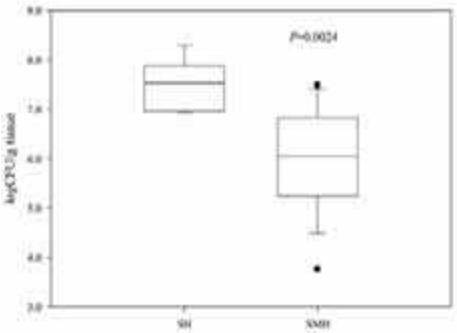

FIG. 3. *H. pylori* colonization in *H. pylori*-infected mice following continuous administration of total mastic extract without the polymer (SMH; $n = 10$ animals) or left untreated (SH; $n = 9$ animals). Viable *H. pylori* counts are expressed in log CFU/g gastric tissue. A moderate 1.5-log reduction in *H. pylori* colonization was observed ($P = 0.0024$) in the SMH study group compared to the SH control group.

parison with data in the literature. The ^{1}H and ^{13}C NMR data for 24*Z*-isomasticadienonic acid have never been reported, and the stereochemistry of the double bond at position 24 of masticadienonic acid, isomasticadienonic acid, masticadienolic acid, and isomasticadienolic acid has never been studied. In all cases, the NOESY correlation of Me-27 with the double bond proton H-24 confirmed the stereochemistry as *Z*. The ^{1}H and ^{13}C NMR data of 24*Z*-isomasticadienonic acid are reported herein for the first time.

In vivo activity of mastic TMEWP against *H. pylori* infection in vivo. During the course of the experiment, none of the participating mice died and no statistical difference was observed between the three groups with regard to animal weight gain (data not shown). Mean water consumption was approximately the same for each animal group and ranged from 4.2 to 4.5 (± 0.1 [standard error of the mean]) ml, regardless of the presence of TMEWP. Average extract consumption was the same for both SM and SMH groups, calculated at 0.75 mg per mouse and taking into account mean water consumption volumes. In the control uninfected group, SM, absence of *H. pylori* infection was confirmed by PCR, serology, or *H. pylori* culture of the gastric samples. In the untreated *H. pylori*-infected SH group, the presence of *H. pylori* was confirmed by PCR and quantitative culturing in 9 out of 10 animals. In the *H. pylori*-negative animal, further analysis of serum anti-*H. pylori* IgG antibodies confirmed the absence of infection and, therefore, it was excluded from the study. The rest of the animals were positive for the presence of *H. pylori* by PCR, and the mean *H. pylori* colonization was calculated at 7.5 ± 0.18 log CFU/g of tissue. In the TMEWP-treated *H. pylori*-infected SMH group, the presence of *H. pylori* was confirmed by PCR in all the animals, and the mean *H. pylori* colonization was calculated at 6.0 ± 0.35 log CFU/g of gastric tissue. A statistically significant reduction in *H. pylori* viable counts was calculated between the two animal groups ($P = 0.0024$) (Fig. 3). These results were also consistent with the histopathologic observations, showing reduced colonization of the bacterium in the gastric antrum

TABLE 1. *H. pylori* colonization in *H. pylori*-infected mice treated with TMEWP

Location	Group	No. of mice of grade[a]:				*P*[b]
		0	1	2	3	
Antrum	SH	1	3	3	2	
	SMH	3	6	1	0	0.031
Corpus	SH	5	4	1	0	
	SMH	10	0	0	0	0.038

[a] Colonization grades are according to the updated Sydney system (11), as follows: normal, 0; mild, 1; moderate, 2; marked, 3.
[b] Statistical analysis with reference to the SH control group, done by Wilcoxon rank sum test. Both correlations shown were significant.

and corpus in the TMEWP-treated SMH group ($P = 0.031$ for antrum, $P = 0.037$ for corpus) (Table 1).

H. pylori-specific IgG antibodies are the dominant antibody class present in the sera of chronically *H. pylori*-infected mice and may serve as an indicator of successful *H. pylori* infection. A significant difference in anti-*H. pylori* IgG antibody titers was observed between *H. pylori*-infected (groups SH and SMH) and noninfected control mice (SM). However, no statistical difference was detected between the TMEWP-treated SMH group and the untreated *H. pylori*-infected SH group with regard to serum anti-*H. pylori* IgG levels (Fig. 4).

Histopathologic evaluation of the gastric mucosa revealed a mild induction of chronic gastritis in the antrum and the corpus fundus in the animals of the untreated *H. pylori*-infected SH group (Tables 2 and 3). Gastric samples were characterized by mild infiltration of the lamina propria with scattered lymphocytes and neutrophils. Chronic inflammatory infiltration in the antrum was mild in eight animals and moderate in one animal. In the corpus, only six animals developed mild chronic inflammatory infiltration (Table 2). The activity of chronic gastritis that developed was mild to moderate (mild in six animals, moderate in two animals) (Table 3) in the antrum and milder in the corpus (normal in seven animals, mild in two animals)

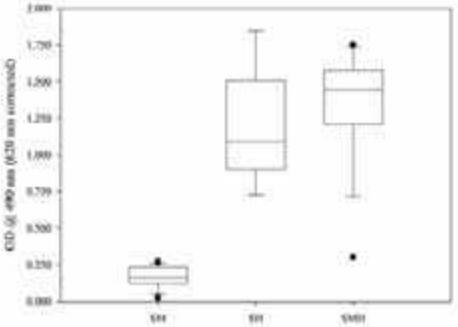

FIG. 4. Serum anti-*H. pylori* IgG antibody response in *H. pylori*-infected animals treated with total mastic extract without the polymer (SMH) or left untreated (SH). Uninfected control mice that received total mastic extract without the polymer are also depicted (SM). Mouse sera were diluted to 1:50. No difference in anti-*H. pylori* titers was observed at 12 weeks postinfection between the SMH and SH groups.

TABLE 2. Chronic inflammatory infiltration[a] in *H. pylori*-infected mice treated with TMEWP

Location	Group	No. of mice of grade[b]:			P[c]
		0	1	2	
Antrum	SH	0	8	1	
	SMH	0	8	2	0.713
Corpus	SH	3	6	0	
	SMH	7	3	0	0.569

[a] Lymphocytic infiltration.
[b] Histopathology grades are according to the updated Sydney system (11) as follows: normal, 0; mild, 1; moderate, 2.
[c] Statistical analysis with reference to the SH control group, done by Wilcoxon rank sum test.

(Table 3). No development of glandular atrophy or intestinal metaplasia was observed, as the time interval from the onset of infection was too short. In the extract-treated *H. pylori*-infected SMH group, the development of chronic gastritis was similar to the SH group (Tables 2 and 3), with marginally reduced numbers of neutrophils infiltrating the lamina propria in some cases. However, statistical analysis with regards to chronic inflammatory infiltration or the development of chronic active gastritis revealed no significant differences between the two animal groups. The collected data from the animal studies suggested that continuous administration of 0.75 mg TMEWP to *H. pylori*-infected mice may moderately reduce *H. pylori* colonizing numbers without a profound effect on the associated gastritis. However, prophylactic administration of TMEWP did not prevent *H. pylori* colonization (data not shown).

In vitro activity of mastic total extract, fractions, and compounds against *H. pylori* strain SS1. Having observed a moderate antimicrobial effect in vivo against *H. pylori*, we proceeded to investigate the potential in vitro anti-*H. pylori* activity of TMEWP and its acidic and neutral fractions against a panel of 10 clinical isolates of *H. pylori* and the CCUG 38771 reference strain (Table 4). Figure 5 depicts characteristic kill curves for strains LAVHP-6 (one of the least susceptible strains) and LAVHP-7 (the most susceptible strain). Mastic extracts exhibited concentration- and strain-dependent bactericidal activities. More specifically, in all strains tested, without exception the acidic fraction exhibited the highest activity, with a mean MBC of 0.136 mg/ml, followed by the TMEWP (MBC, 0.256 mg/ml). Reduced activity was observed for the neutral fraction of the TMEWP (0.638 mg/ml). Up to twofold differences were observed in the MBC between individual strains tested, and

TABLE 3. Activity of chronic gastritis[a] in *H. pylori*-infected mice treated with TMEWP

Location	Group	No. of mice of grade[b]:			P[c]
		0	1	2	
Antrum	SH	1	6	2	
	SMH	5	2	3	0.369
Corpus	SH	7	2	0	
	SMH	8	1	1	0.967

[a] Neutrophil infiltration.
[b] Histopathology grades are according to the updated Sydney system (11) as follows: normal, 0; mild, 1; moderate, 2.
[c] Statistical analysis with reference to the SH control group, done by Wilcoxon rank sum test.

TABLE 4. Minimum bactericidal concentrations of mastic extracts[a] on *H. pylori* strains

Strain	MBC (mg/ml)		
	Total extract	Acidic fraction	Neutral fraction
CCUG 38771	0.390	0.240	1.560
LAVHP-1	0.195	0.120	0.390
LAVHP-2	0.195	0.120	0.780
LAVHP-3	0.195	0.120	0.780
LAVHP-4	0.390	0.120	0.780
LAVHP-5	0.195	0.120	0.390
LAVHP-6	0.390	0.120	0.780
LAVHP-7	0.090	0.060	0.390
LAVHP-8	0.390	0.120	0.390
LAVHP-9	0.195	0.240	0.390
LAVHP-10	0.195	0.120	0.390

[a] With the exception of the acidic fraction, for which successive twofold dilutions ranged from 0.060 to 1.920 mg/ml, all other extracts were tested at a final concentration range of 0.049 to 1.560 mg/ml.

only in the case of LAVHP-7 strain was a higher susceptibility against the TMEWP and its acidic fraction observed.

Having obtained the highest activity with the acidic fraction of the TMEWP, we proceeded to test the isolated pure acidic compounds for anti-*Helicobacter* activity. Highest overall activity was obtained consistently and for all 11 of the *H. pylori* strains tested with isomasticadienolic acid, with a mean MBC of 0.202 mg/ml (0.443 mM), followed by masticadienolic (0.220 mg/ml [0.482 mM]), oleanonic (0.292 mg/ml [0.643 mM]), and moronic acid (0.310 mg/ml [0.683 mM]) (Table 5). Interestingly, the 3-oxo derivatives, isomasticadienonic and masticadienonic acids, showed reduced activity compared to the corresponding 3-hydroxyl derivatives.

DISCUSSION

All previous in vivo studies evaluating activity of mastic gum against *H. pylori* have used a crude mastic preparation which contained a high percentage (30%) of an insoluble and sticky polymer. We speculated that the presence of the polymer hindered potential in vivo activity of mastic during oral administration, and for this reason we prepared TMEWP. We have verified that the chemical consistency of TMEWP was virtually identical to that of crude mastic gum, except for the absence of the polymer, and it also presented better solubility properties and increased concentration of active constituents. Previous animal studies for the determination of the potential anti-*Helicobacter* activity of mastic gum were organized on a short-term administration schedule. However, we have extended our administration and hence tested the TMEWP activity *over* a period of 3 months.

In the present study we utilized an established *H. pylori* infection model to evaluate the potential therapeutic effect of continuous TMEWP administration on *H. pylori* colonization and development of associated gastritis. This model involves the mouse-adapted *H. pylori* Sydney strain 1 (SS1), which colonizes the C57BL/6 mouse heavily and leads to the development of appreciable levels of gastritis closely mimicking human *H. pylori* infection (16, 19, 31). The particular infection model

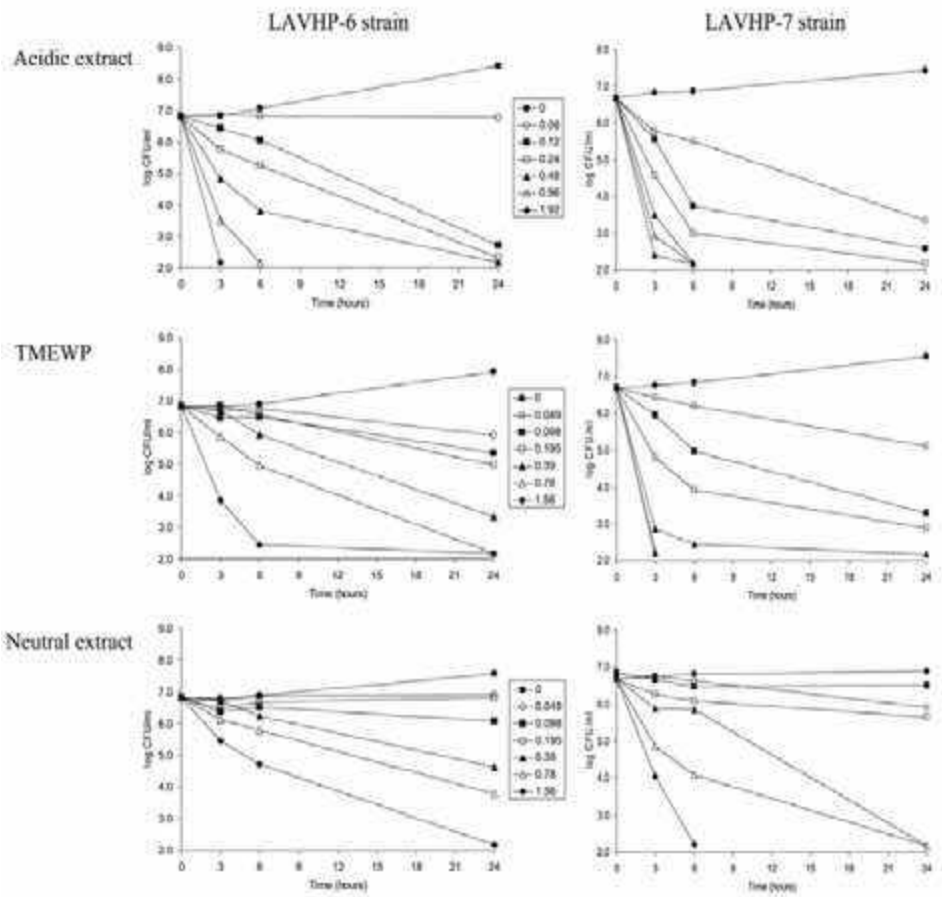

FIG. 5. Bactericidal activity of mastic gum extracts against *H. pylori* in a liquid medium. *H. pylori* strains LAVHP-6 (more resistant strain) and LAVHP-7 (most susceptible strain) were cultured under microaerophilic conditions in BHI as described in Materials and Methods and exposed to acidic, TMEWP, and neutral fractions at the concentrations depicted in the legend. After further incubation, viability was determined at each time point.

TABLE 5. Mean MBC of mastic triterpenic acid compounds within the mastic acidic fraction against *H. pylori*[a]

Substance	Mean MBC in μg/ml (mM)
24*Z*-Isomasticadienonic acid	0.333 (0.733)
24*Z*-Masticadienonic acid	0.350 (0.770)
24*Z*-Isomasticadienolic acid	0.202 (0.443)
24*Z*-Masticadienolic acid	0.220 (0.482)
Oleanonic acid	0.292 (0.643)
Moronic acid	0.310 (0.683)

[a] *H. pylori* strains are described in Table 4.

has been successfully utilized in the past to evaluate potential anti-*H. pylori* activity of a number of agents, such as antibiotics (14, 15) or lactic acid-producing bacteria (27, 28).

Our experiments showed that the mastic total extract could moderately reduce *H. pylori* colonization in the antrum and corpus of the stomach. The reduction in colonization levels calculated was approximately 30-fold, in the range of 1.5 log CFU/g of tissue. These results were in concurrence with the visible reduction in *H. pylori* colonization observed in the histopathology evaluations. However, such a moderate fall in *H. pylori* colonizing numbers could not support any attenuation of the *H. pylori*-associated chronic active gastritis. We have documented no such decline in either neutrophilic or lymphocytic

infiltration within the lamina propria. Equally, no reduction was observed with reference to serum anti-*H. pylori* IgG antibodies between the mastic-treated and nontreated animal groups. Collectively, these results are in line with earlier observations involving the same infection model, in which 7-day monotherapy with crude mastic tear diluted in 100% ethanol failed to eradicate *H. pylori* (17). Nevertheless, we were able to document a moderate drop in colonization levels possibly by increasing the bioavailability of the active mastic constituents, following polymer removal. Another main advantage of our study was the application of a longer period of administration and observation. In this way we were able to administer 0.75 mg of TMEWP (the approximate equivalent of 1 mg total mastic gum) continuously through the animal water supply, for a period of 3 months, as opposed to only 1-week therapy regimens reported in other studies. Our mode of administration mimicked more closely real life conditions, where active mastic constituents can be released in a sustained release mode following daily consumption of mastic gum. Although 3 months proved too short a period for the mastic total extract to eradicate *H. pylori*, results suggest that in real life, habitual daily consumption of mastic gum over a much longer period may well create conditions favoring a decrease in *H. pylori* colonization levels. However, we observed no prophylactic activity of TMEWP on *H. pylori* infection, although our observations depend on a small number of animals. Equally, to date no epidemiological data exist to support such a prophylactic effect among the mastic gum-consuming population of Chios Island.

A detailed in vitro investigation was performed in order to identify the potentially most active fractions in mastic extracts. The TMEWP was further separated into an acidic and a neutral fraction, and the antimicrobial activities of these fractions, as well as that of mastic total extract, were determined. We observed a moderate activity for the total mastic extract against a panel of 11 *H. pylori* strains at a mean MBC of 0.256 mg/ml, which is higher than that previously reported against the *H. pylori* SS1 strain for mastic gum preparations diluted in ethanol (17). This could well be attributed to differences in methodology and the differential susceptibility of diverse *H. pylori* isolates, as our in vitro data clearly demonstrated. The acidic fraction of the TMEWP was found to be the most active, with an MBC as low as 0.136 mg/ml, consistently in all strains tested. In order to identify the most active constituent among the contained triterpenic acids, the acid fraction was further submitted to several chromatographic separations that afforded the six major constituents. They were all tested against the same *H. pylori* strains as mastic extracts, and the most active one was found to be isomasticadienolic acid. None of the pure compounds was more active than the whole acid fraction, suggesting that, although the anti-*H. pylori* activity of mastic could be located particularly in the acidic fraction, probably the final activity is a result of synergy between all the acid constituents. Although it is the first case in which specific mastic acid triterpenic compounds have been shown to exhibit anti-*H. pylori* activity, other tetracyclic acids similar to those contained in mastic have been reported to be active against *Escherichia coli* (35). In our study, however, due to limitations in the isolation and purification steps, we were not able to test the potential antimicrobial in vivo activity of individual triterpenic compounds.

Emerging antibiotic resistance and reduced patient compliance are the main reasons for failed *H. pylori* eradication therapy. In addition, the considerable expense of the antibiotic regimen coadministered with a proton pump inhibitor creates the need for alternative antibiotics for combination therapy. The present study demonstrated that a mastic gum extract without the polymer constituent poly-β-myrcene was effective in reducing *H. pylori* colonization levels by 30-fold in infected mice over an administration period of 3 months and that the activity could be attributed to triterpenic acids within the acid fraction of mastic extracts. These results suggest that removal of the constituent poly-β-myrcene polymer can produce an enhanced therapeutic moiety which may exhibit anti-*H. pylori* activity, detectable over a shorter time period, bearing all the advantages of a nutritional product. We plan to evaluate further the true therapeutic potential of the acidic and neutral fractions of mastic gum in extensive animal studies following administration of the respective triterpenic constituents, alone and in combination, to assess synergic effects. The role of such mastic constituents will also be assessed with reference to potential synergy with currently used antibiotic eradication schemes.

Finally, our results also suggest that habitual long-term mastic consumption may be effective in moderating *H. pylori* colonization, although to date no epidemiological data exist to support the hypothesis of a reduced prevalence of *H. pylori* infection among the mastic gum-consuming population of Chios Island.

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The Long-term Impact of *Helicobacter pylori* Eradication on Gastric Histology: a Systematic Review and Meta-analysis

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Keywords
eradication, gastric atrophy, intestinal metaplasia, meta-analysis.

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Summary

Background: *Helicobacter pylori* infection is a crucial factor in the multistep carcinogenic process of gastric cancer. In this process the gastric mucosa evolves through the stages of acute gastritis, chronic gastritis, gastric atrophy (GA), and intestinal metaplasia (IM) before developing gastric adenocarcinoma.
Aims: The main aim of this study was to systematically review the long-term effects of *H. pylori* eradication on gastric histology (i.e. effects on GA and IM for both antrum and corpus) by meta-analyzing all relevant studies.
Methods: Extensive English-language medical literature searches for human studies were performed through October 2006, using suitable key words. Pooled estimates [odds ratio (OR) with 95% confidence intervals (CI)] were obtained using random-effects model.
Results: For antrum GA the pooled OR with 95% CI was 0.554 (0.372–0.825), $p = 0.004$. For corpus GA the pooled OR was 0.209 (0.081–0.538), $p < 0.001$. For antrum IM the pooled OR was 0.795 (0.587–1.078), $p = 0.14$. For corpus IM the pooled OR was 0.891 (0.663–1.253), $p = 0.506$.
Conclusion: The results showed significant improvement of GA, whereas improvement was not shown for IM.

Gastric cancer remains a leading worldwide health problem. It continues to be the second most common cause of cancer-related mortality in the world, with an estimated 700,000 deaths annually. It is now the fourth most common cancer overall, behind only cancers of lung, colon, and breast [1].

Helicobacter pylori infection has been cited as a gastric carcinogen for more than 10 years [2] and recently Marshall and Warren were awarded the 2005 Nobel Prize in Medicine and Physiology for their discovery of *H. pylori* and its causative role in gastric pathology including gastric cancer. In the developing world, most people acquire the infection during childhood and develop persistent inflammation in their stomachs, which lasts for decades [3,4]. Gastric cancer, at least of intestinal type, is believed to arise via a multistep process that evolves through the stages of active inflammation (AI), chronic inflammation (CI), gastric atrophy (GA), intestinal metaplasia (IM), and dysplasia before developing gastric adenocarcinoma [5]. This notion is supported by data which strongly suggests that GA and IM are important predictors of gastric cancer [6–9].

Taking into account that *H. pylori* is an important causative factor in gastric carcinogenesis, it is logical to assume that its eradication may have an important role to play in the prevention of gastric cancer [10,11]. This assumption is enhanced by the report by Uemura et al. [12] that eradication of *H. pylori* even prevents recurrence of early gastric cancer after mucosal resection, suggesting that the emergence of gastric cancer can be prevented by *H. pylori* eradication. However, treatment of *H. pylori* infection might not be beneficial if therapy is given to at-risk individuals beyond the "point of no return" when the development of malignancy would progress in an unrelenting fashion despite cure of the infection. So far, it remains unclear whether the development of GA and IM of the stomach represent this point of no return and it is of fundamental importance therefore to answer the question of whether these lesions are reversible, because if this is the case, then therapeutic intervention may be possible, but if not, efforts can only be directed at preventing the development of these lesions by treating the infection in childhood.

This systematic review and meta-analysis examined the long-term impact of *H. pylori* eradication on gastric histology and in particular aimed at testing the hypothesis that curing *H. pylori* infection reverses the precancerous lesions of GA and IM. The study is justified as so far no similar meta-analysis has been published.

Methods

Data Identification and Extraction

We searched the MEDLINE/PUBMED database up to October 2006 to identify all relevant English-language medical literature for human studies under the search text terms: *Helicobacter pylori* and atrophy or intestinal metaplasia OR gastritis and treatment or eradication. We also performed a full manual search of all review articles, recently published editorials, and retrieved original studies. Data were extracted independently from each study by two of the authors (T.R. and D.P.) by using a predefined form, and disagreements were resolved by discussion with a third investigator and consensus.

Selection Criteria

Inclusion and exclusion criteria were delineated before the commencement of the literature search. Thus, eligible studies published as full articles were included in this systematic review if they met all the following criteria: 1, patients to be adults, i.e. over 19 years of age; 2, gastric histology to be investigated separately for antrum and corpus; 3, in all studies a similar methodology for histology scoring, i.e. the updated Sydney system [13], to have been utilized; 4, subjects to have been studied by histology at baseline and at least 12 months later; 5, at histology, subjects apart from GA and IM to have no malignant mucosal lesions, i.e. dysplasia or cancer; and 6, subjects not to be on long-term PPI treatment. Studies not meeting the aforementioned criteria and in addition studies without data for retrieval and duplicate publications were excluded. When two papers reported the same study on histologic changes, the publication that was more informative was selected.

Statistical Analysis

Agreement on the selection of studies between the two reviewers was evaluated by the κ coefficient. We calculate the pooled odds ratios (ORs) and 95% confidence intervals (CI) and compared outcomes of individual studies by using the DerSimonian and Laird method (random effect model) [14]. Forest plots were constructed for visual display of log ORs of individual studies. Heterogeneity between studies was evaluated with the Cochran *Q*-test [15] and it was considered to be present if the *Q*-test provided a *p* value of less than 0.10 [16]. In the presence of significant statistical heterogeneity, sensitivity analyses were performed to search for the possible sources such as sample size of each study, duration of the follow up, number of the biopsy samples taken, etc. These analyses were achieved by repeating the meta-analyses with exclusion of each individual study one at a time, in order to assess the overall effect of each study on the pooled ORs [17]. This indicates which particular studies are most influential and might help in the evaluation of the possibility that the conclusions result from the influence of a particular study. The likelihood of publication bias was assessed by constructing funnel plots [17,18], and their symmetry was estimated by the Begg and Mazumdar adjusted rank correlation test [19]. Data, in various formats, i.e. as dichotomous (number of events), as continuous (means or medians) and as computed effect sizes, were meta-analyzed by choosing the most suitable data entry option to the meta-analysis software used (Comprehensive Meta Analysis – Version 2, BIOSTAT INC., Englewood, NJ, USA).

Results

Descriptive Assessment and Study Characteristics

A flow chart describing the process of study selection is shown in Fig. 1. Out of 1153 titles initially generated by the literature searches, 1005 were rejected as the title suggested that the articles were not appropriate. Of the remaining 148 abstracts, 117 were excluded (reviews, editorials, duplications, not English language, etc.). Therefore, 31 papers remained candidates for eligibility. Of these, 23 were rejected on the basis of not fulfilling the inclusion criteria. Therefore, eight studies remained eligible for meta-analysis [20–27]. Initial agreement between the reviewers for the selection of relevant articles was high (κ = 0.94).

The main characteristics of the papers eligible for meta-analysis are shown in Table 1. Of these, one was a randomized control study [20] and the rest were observational studies [21–27]. They were conducted in different parts of the world and all were single-centre studies. Not all the papers gave data for the two histologic parameters evaluated, i.e. GA and IM separately for gastric corpus and antrum. Table 1 shows which of these parameters was studied by each paper. There were two papers [28,29] that did not evaluate GA and IM separately for gastric corpus and antrum and instead provided results for the whole of the stomach. Therefore, these two studies were not included in this meta-analysis. Similarly, two papers [30,31] that studied patients on PPIs were also not included.

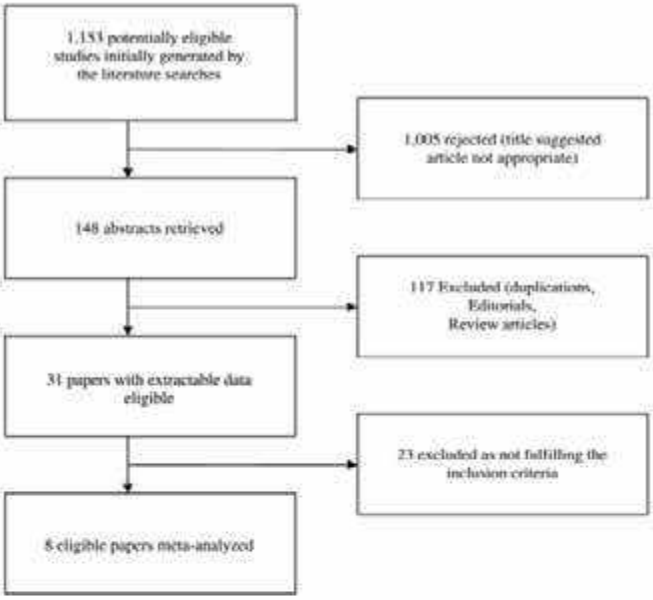

Figure 1 Flow diagram of the studies identified in this systematic review and meta-analysis.

Table 1 Main characteristics of the eight studies selected for meta-analysis

Reference No.	Study name (Country)	Study arms (n)		Follow-up (months)	Histology assessment	Histologic parameters evaluated			
		Eradication	Noneradication			GA	IM	GA	IM
20	Sung et al. 2000 (China)	(226)	(245)	12	Updated Sydney System	Yes	Yes	Yes	Yes
21	Kim et al. 2000 (Korea)	(41)	(16)	24	Updated Sydney System	No	Yes	No	Yes
22	Ohkusa et al. 2001 (Japan)	(115)	(48)	12–15	Updated Sydney System	Yes	Yes	Yes	Yes
23	Ruiz et al. 2001 (Colombia)	(29)	(21)	72	Updated Sydney System	Yes	No	No	No
24	Ito et al. 2002 (Japan)	(22)	(22)	60	Updated Sydney System	Yes	Yes	Yes	Yes
25	Yamada et al. 2003 (Japan)	(87)	(29)	10–50	Updated Sydney System	Yes	Yes	Yes	Yes
26	Lahner et al. 2005 (Italy)	(38)	(36)	48–137	Updated Sydney System	Yes	Yes	Yes	Yes
27	Lu et al. 2005 (China)	(92)	(87)	36	Updated Sydney System	Yes	Yes	No	No

Gastric Atrophy

The ORs with 95% CIs of GA between *H. pylori* treated and untreated groups in individual studies and pooled OR for gastric antrum and corpus are displayed in forest plots in Figs 2 and 3.

For antrum GA the pooled OR with 95% CI was 0.554 (0.372–0.825) with test for overall effect $Z = -2.91$ and $p = 0.004$. There was significant heterogeneity among these trials with Q-value of 14.83, $I^2 = 59.55$, $p = 0.022$. Because of this we performed multiple sensitivity analyses in which each study was excluded individually, one at a time, to examine whether heterogeneity and pooled estimates would be affected. We found that the pooled OR was not influenced and therefore no study was excluded from the meta-analysis. There was no publication bias (two-tailed

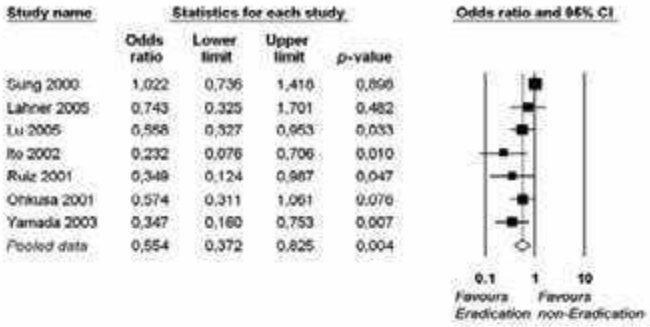

Figure 2 Forest plot of studies comparing antrum gastric atrophy in eradicated versus noneradicated patients.

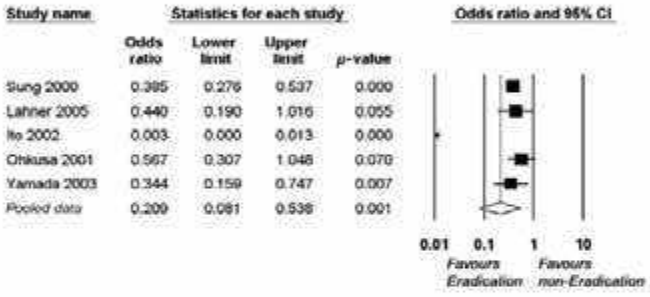

Figure 3 Forest plot of studies comparing corpus gastric atrophy in eradicated versus noneradicated patients.

p value = 0.133) and the respective funnel plot is shown in Fig. 4.

For corpus GA the pooled OR was 0.209 (0.081–0.538), $Z = -3.24$, $p < 0.001$. There was significant heterogeneity among these trials with Q-value of 34.37, $I^2 = 89.3$, $p < 0.001$. To explore this we performed multiple sensitivity analyses, which showed that the pooled OR was not influenced and therefore no study was excluded from the meta-analysis. There was no publication bias ($p = 0.221$).

Intestinal Metaplasia

The ORs with 95% CIs of IM between *H. pylori* treated and untreated groups in individual studies and pooled OR for gastric antrum and corpus are displayed in forest plots in Figs 5 and 6.

For antrum IM the pooled OR was 0.795 (0.587–1.078), $Z = -1.476$, $p = 0.14$. There was no heterogeneity ($Q = 9.068$, $I^2 = 33.83$, $p = 0.17$) and in addition there was no publication bias ($p = 0.368$).

For corpus IM the pooled OR was 0.891 (0.663–1.253), $Z = -0.665$, $p = 0.506$. As in antrum IM there was no heterogeneity among meta-analyzed studies (Q-value 7.37, $I^2 = 32.22$, $p = 0.194$) and there was also no publication bias ($p = 0.06$).

Discussion

The effect of *H. pylori* eradication on premalignant lesions such as GA and IM of the stomach has intrigued many investigators worldwide. However, conflicting messages have been reported in previous studies. Some years ago Hojo et al. reviewed the alteration of gastric histology after cure of *H. pylori* infection [32]. However, this was a literature survey without meta-analysis. Moreover, since then more studies have been published and it is still disputed whether GA and IM improve after cure of *H. pylori* infection.

In this meta-analysis we pooled the data of various published studies in an effort to examine the alteration of histology and especially to answer the crucial question of whether GA and IM of the stomach are reversible after *H. pylori* eradication, because if this is the case, then therapeutic intervention may be possible, but if not, efforts can only be directed at prevention.

The results indicated that *H. pylori* eradication has beneficial long-term effects on GA but not on IM at both

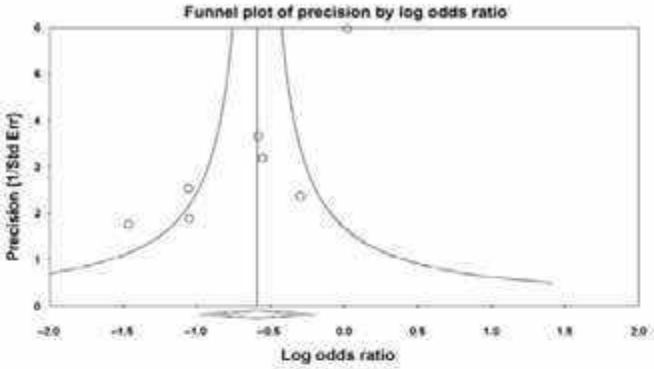

Figure 4 Funnel plot analysis comparing antrum gastric atrophy in eradicated and noneradicated patients. Statistical analysis confirmed no evidence of publication bias. Similarly, no evidence of publication bias was found for the other histological parameters studied, i.e. corpus gastric atrophy, antral intestinal metaplasia, corpus intestinal metaplasia.

Study name	Odds ratio	Lower limit	Upper limit	p-value
Sung 2000	0.965	0.695	1.340	0.832
Lahner 2005	0.700	0.306	1.604	0.399
Lu 2005	1.268	0.762	2.210	0.337
Ito 2002	0.677	0.231	1.983	0.476
Ohkusa 2001	0.446	0.240	0.826	0.010
Yamada 2003	0.844	0.394	1.810	0.663
Kim 2000	0.441	0.153	1.270	0.129
Pooled data	0.799	0.587	1.078	0.140

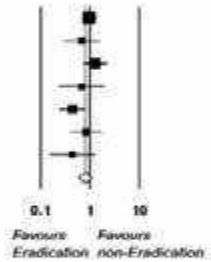

Figure 5 Forest plot of studies comparing antrum intestinal metaplasia in eradicated versus noneradicated patients.

Study name	Odds ratio	Lower limit	Upper limit	p-value
Sung 2000	1.167	0.841	1.621	0.355
Lahner 2005	1.030	0.451	2.355	0.944
Ito 2002	0.453	0.153	1.340	0.152
Ohkusa 2001	1.214	0.659	2.237	0.534
Yamada 2003	0.592	0.275	1.273	0.180
Kim 2000	0.441	0.153	1.270	0.129
Pooled data	0.891	0.633	1.253	0.500

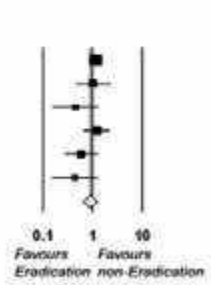

Figure 6 Forest plot of studies comparing corpus intestinal metaplasia in eradicated versus noneradicated patients.

anatomic sites studied, i.e. antrum and corpus. The interpretation of this finding is not clear, but several hypothetical arguments may explain the irreversibility of this histologic change after *H. pylori* eradication [32]. Thus, fibrotic changes will not regress as cutaneous scars do not disappear, and antibodies against *H. pylori* may cross-react with glandular cells after the cure of *H. pylori* infection. In addition, it may be possible that bile reflux and bacteria

other than *H. pylori* cause epithelial damage. Also, certain types of diet and the patient's age may strongly influence the development of IM in the gastric mucosa.

Whatever the interpretation of the findings of our study, the results suggest that treatment of *H. pylori* infection in adulthood is not beneficial in reversing IM. It seems therefore, that the development of IM in the stomach is not reversible by *H. pylori* eradication and possibly represents the "point of no return" beyond which the development of malignancy may progress in an unrelenting fashion despite cure of the infection. Therefore, efforts should be directed at preventing the development of such a lesion by treating the infection early in life.

This meta-analysis suffers from some inherent weaknesses: the most important being underpowered studies of small number. In addition, there are some methodological flaws that might affect the results. These flaws may include factors such as number of biopsy samples taken, the method of histologic classification of findings, sample size of each study, and duration of follow-up. To alleviate such influences and in order to unify the method of histologic evaluation of biopsy samples, we selected only reports employing the updated Sydney System. In addition, in order to further explore the possible influences of the previously mentioned factors on the results, we performed sensitivity analyses, which showed that the pooled ORs were not significantly influenced by such factors. One might argue that in addition to the previously discussed, the results might have been influenced by factors other than methodological biases. For example, the inability to retrieve unpublished studies is a drawback of this study. We were not able to retrieve published abstracts because of the absence of such a searching mechanism. However, we maximized the chances of detecting such studies by going through the references of the selected articles.

In summary, the results of this systematic review and meta-analysis suggest that cure of *H. pylori* infection has beneficial long-term effects on GA but not on IM. However, the follow-up periods of the meta-analyzed studies are far too short in a long process of mucosal carcinogenesis. Clearly, more studies, especially RCTs, with a longer follow-up period are necessary to assess the long-term benefit of the treatment and whether cure of *H. pylori* infection may confer a benefit in halting disease progression.

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Relationship Between *Helicobacter pylori* Infection and Esophageal Neoplasia: A Meta-analysis

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Background & Aims: ***Helicobacter pylori* is an important causative factor in gastric carcinogenesis. However, its role in extragastric gastrointestinal malignancies, such as esophageal cancer, is controversial. The aim of this study was to explore the relationship of *H pylori* infection and *H pylori cagA*-positive strain with this malignancy by performing meta-analysis of all relevant studies.** ***Methods:*** **Extensive MEDLINE English language medical literature searches for human studies were performed through February 2007 with suitable keywords. Pooled estimates were obtained by using fixed or random-effects model as appropriate. Heterogeneity between studies was evaluated with the Cochran Q test, whereas the likelihood of publication bias was assessed by constructing funnel plots. Their symmetry was estimated by the Begg and Mazumdar adjusted rank correlation test.** ***Results:*** **In adenocarcinoma patients there were inverse significant relationships with both the *H pylori* prevalence (pooled odds ratio [OR], 0.52; 95% confidence interval [CI], 0.37–0.73; $P < .001$) and the prevalence of *H pylori cagA*-positive strain (pooled OR, 0.51; 95% CI, 0.31–0.82; $P = .006$). Similarly in patients with Barrett's esophagus there were inverse significant relationships (pooled OR, 0.64; 95% CI, 0.43–0.94; $P = .025$ and pooled OR, 0.39; 95% CI, 0.21–0.76; $P = .005$, respectively). In patients with squamous cell carcinoma there were no significant relationships with both *H pylori* prevalence (pooled OR, 0.85; 95% CI, 0.55–1.33; $P = .48$) and the prevalence of *H pylori cagA*-positive strains (pooled OR, 1.22; 95% CI, 0.7–2.13; $P = .48$).** ***Conclusions:*** **The results showed an inverse statistically significant relationship of *H pylori* infection with both esophageal adenocarcinoma and Barrett's esophagus, which might suggest a protective role of the infection in these entities. On the contrary, no statistically significant relationship with squamous cell carcinoma was found.**

Since the discovery of *Helicobacter pylori* by Marshall and Warren,[1] overwhelming epidemiologic evidence has shown that populations infected with *H pylori* have an increased risk for gastric cancer when compared with uninfected control populations.[2–4] In addition, infection with *cagA*-positive strains of *H pylori* increases the risk associated with *H pylori* infection alone.[5]

The incidence of esophageal adenocarcinoma (AC) has increased in several Western countries.[6–10] It has been suggested that this increase might be linked to declining rates of *H pylori* infection in Western society.[11] However, the role of *H pylori* infection in esophageal neoplasia is controversial because both positive and inverse associations have been reported in Barrett's esophagus (BE) and esophageal cancer (AC and squamous cell carcinoma [SCC]). The aim of this study therefore was to explore the relationship between *H pylori* infection and esophageal pre-neoplastic (BE) and neoplastic disease (AC and SCC) by meta-analysis of all relevant cohort and case-control studies. In addition, we examined the role of *H pylori cagA*-positive strains in these entities. This study was justified by the fact that, so far, no meta-analysis has been published examining the relationship between *H pylori* infection and esophageal neoplasia.

Material and Methods

Data Identification and Extraction

We searched the PubMed, MEDLINE, and Embase databases through February 2007 to identify all relevant English language medical literature for human studies under the search text terms *helicobacter pylori* AND (esophageal cancer OR esophageal neoplasms OR Barrett's esophagus OR adenocarcinoma OR squamous cell carcinoma). We also performed a full manual search of all review articles, recently published editorials, and retrieved original studies. Data were extracted independently from each study by two of the authors (T.R and D.P) by using a predefined form, and disagreements were resolved by discussion with a third investigator and consensus.

Selection Criteria

Inclusion and exclusion criteria were delineated before the commencement of the literature search. Thus, eligible studies were included in this meta-analysis if they met all the following criteria: (1) published as full articles; (2) written in English; (3) to be cohort or case-control studies with raw data on *H pylori* and/or *cagA*-positive *H pylori* strain prevalence in AC, BE, or SCC; (4) studies to be conducted in adults; and (5) *H pylori* infection to be confirmed by serology and/or histology. Studies not meeting the aforementioned criteria and, in addition, studies without data for retrieval and duplicate publications were excluded. When 2 articles reported the same study, the publication that was more informative was selected. In this meta-analysis BE patients with high-grade dysplasia were

Abbreviations used in this paper: AC, adenocarcinoma; BE, Barrett's esophagus; CI, confidence interval; df, degrees of freedom; OR, odds ratio; SCC, squamous cell carcinoma.

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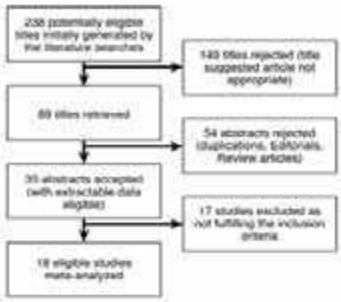

Figure 1. Flow diagram of the studies identified in this meta-analysis.

grouped with patients with esophageal AC, because this grouping was found in the majority of the relevant meta-analysis articles.

Statistical Analysis

Agreement on the selection of studies between the 2 reviewers was evaluated by the κ coefficient. We calculated the pooled odds ratios (ORs) and 95% confidence intervals (CIs) and compared outcomes of individual studies by using the fixed effects model[12] (Mantel-Haenszel method), unless significant heterogeneity was present, where the random effects model[13] was used (DerSimonian and Laird method). Forest plots were constructed for visual display of ORs of individual studies. Heterogeneity between studies was evaluated with the Cochran Q test,[14] and it was considered to be present if the Q test provided a P value of less than .10.[15] In the presence of significant statistical heterogeneity, sensitivity analyses were performed to exclude any possible influence of a single study. These analyses were achieved by repeating the meta-analyses with exclusion of each individual study one at a time to assess the overall effect of each study on the pooled ORs.[16] This indicates which particular studies are most influential and might help in the evaluation of the possibility that the conclusions result from the influence of a particular study. The likelihood of publication bias was assessed by constructing funnel plots that were obtained by plotting the log ORs versus precision (1/SE) of individual studies.[16–18] Their symmetry was estimated by the Begg and Mazumdar adjusted rank correlation test.[18] All analyses were performed by using Comprehensive Meta-analysis software (Version 2; BIOSTAT INC., Englewood, NJ).

Results

Descriptive Assessment and Study Characteristics

A flow chart describing the process of study selection is shown in Figure 1. Of 238 titles initially generated by the literature searches, 18 studies, of which 9 were case-control and 9 cohort studies, remained eligible for meta-analysis.[17–34] Initial agreement between the reviewers for the selection of relevant articles was high (κ = 0.94, 95% CI, 0.86–1).

In the 18 meta-analysis studies there were 22 sets of data comparing *H pylori* and *H pylori cagA*-positive strain prevalence between patients and controls. The main characteristics of the articles eligible for meta-analysis are shown in Tables 1 and 2 (see supplementary material online at www.cghjournal.org). They were conducted in different parts of the world and contained 3262 patients and 7206 controls.

Helicobacter pylori *Prevalence*

Esophageal adenocarcinoma. In the 10 studies examining AC patients, the prevalence of *H pylori* was significantly lower in patients than in controls (253/737 [34.3%] vs 1398/2788 [50.1%], respectively; pooled OR, 0.52; 95% CI, 0.37–0.73; Z value = −3.75; P < .001 [random effects model, Figure 2]). There was no heterogeneity between studies (heterogeneity Q value = 13.7; df (Q) = 9; I^2 = 34.56; P = .13), and also there was no publication bias (Begg and Mazumdar adjusted rank correlation test two-tailed P value = .37) (Figure 3).

Barrett's esophagus. In the 7 studies examining patients with BE, the prevalence of *H pylori* in patients was significantly lower than in controls (796/1621 [49.1%] vs 861/1491

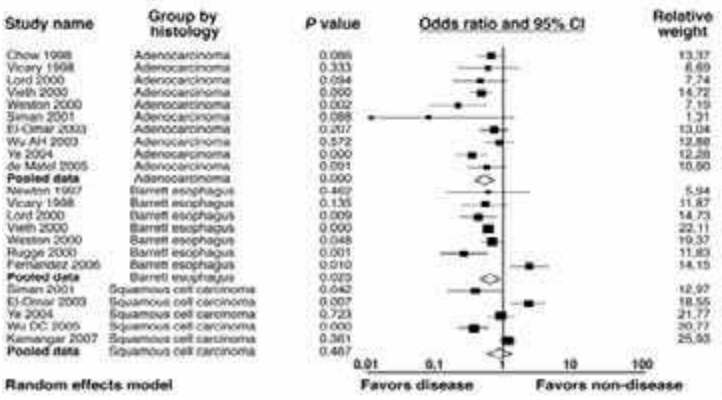

Figure 2. Forest plot showing individual and pooled ORs (95% CIs) in studies (grouped by histology) comparing the *H pylori* prevalence in patients and controls.

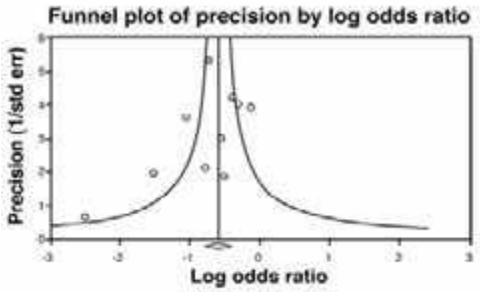

Figure 3. Funnel plot of selected studies examining *H pylori* prevalence in esophageal AC patients and controls. No evidence of publication bias (P = .37, by Begg and Mazumdar adjusted rank correlation test).

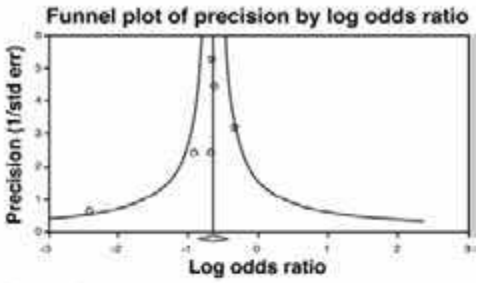

Figure 5. Funnel plot of selected studies examining the *H pylori cagA*–positive strain prevalence in esophageal AC patients and controls. No evidence of publication bias (P = .26, by Begg and Mazumdar adjusted rank correlation test).

[57.7%], respectively; pooled OR, 0.64; 95% CI, 0.43–0.94; Z value = −2.25; P = .025 [random effects model, Figure 2]). There was no publication bias (Begg and Mazumdar adjusted rank correlation test two-tailed P value = 1), but there was heterogeneity between studies (Q value = 22.18; *df* (Q) = 6; I^2 = 72.95; P = .001). For this reason sensitivity analyses were performed to exclude any possible influence of a single study. It was found that the removal of one study[32] had a significant effect on the pooled ORs.

Squamous cell carcinoma. In the 5 studies examining patients with SCC, the prevalence of *H pylori* in patients did not differ significantly from controls (346/600 [57.6%] vs 1083/1874 [57.8%], respectively; pooled OR, 0.86; 95% CI, 0.56–1.33; Z = −0.67; P = .48 [random effects model, Figure 2]). There was heterogeneity between studies (Q value = 26.1; *df* (Q) = 4; I^2 = 84.68; P < .001) but no publication bias (Begg and Mazumdar adjusted rank correlation test two-tailed P value = .8).

Helicobacter pylori *cagA–Positive Strain Prevalence*

Esophageal adenocarcinoma. In the 6 studies examining AC patients, the prevalence of the *H pylori cagA*–positive strain was significantly lower in patients than in controls (120/462 [26%] vs 774/1936 [40%], respectively; pooled OR, 0.51; 95% CI, 0.31–0.82; Z value = −2.77; P = .006 [random effects model, Figure 4]). There was no heterogeneity between studies (Q value = 2.78; *df* (Q) = 5; I^2 = 0.00; P = .73), and also there was no publication bias (Begg and Mazumdar adjusted rank correlation test two-tailed P value = .26) (Figure 5).

Barrett's esophagus. In the 5 studies examining patients with BE, the prevalence of the *cagA*-positive *H pylori* strain was significantly lower than in controls (87/244 [35.6%] vs 185/359 [51.5%], respectively; pooled OR, 0.39; 95% CI, 0.21–0.76; Z value = −2.78; P = .005 [random effects model, Figure 4]). There was no publication bias (Begg and Mazumdar adjusted rank correlation test two-tailed P value = .806), but there

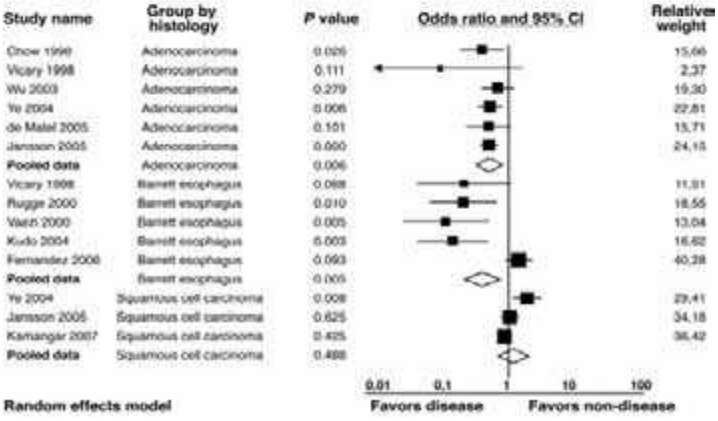

Figure 4. Forest plot showing individual and pooled ORs (95% CIs) in studies (grouped by histology) comparing the *H pylori cagA*-positive strain prevalence in patients and controls.

was heterogeneity between studies (Q value = 26.9; *df* (Q) = 4; I^2 = 85.13; $P < .001$). For this reason, sensitivity analyses were performed to exclude any possible influence of a single study. It was found that the removal of one study[32] had a significant effect on the pooled ORs.

Squamous cell carcinoma. In the 3 studies examining patients with SCC, the prevalence of *H pylori* did not differ significantly in comparison with controls (304/587 [51.7%] vs 1138/2311 [49.2%], respectively; pooled OR, 1.22; 95% CI, 0.7–2.13; Z value = 7; $P = .48$ [random effects model, Figure 4]). There was heterogeneity between studies (Q value = 7.52, *df* (Q) = 2; I^2 = 73.4; $P = .023$) but no publication bias (Begg and Mazumdar adjusted rank correlation test two-tailed P value = .3).

Discussion

In this study we pooled the data of published studies in an effort to examine the relationships between *H pylori* infection and esophageal neoplasia by meta-analysis of all relevant cohort and case-control studies.

The results showed an inverse relationship of *H pylori* as well as the *H pylori cagA*–positive strain prevalence with both BE and AC, suggesting that *H pylori* infection might play a protective role in this type of esophageal malignancy. The results are in accordance with those of a recent systematic review and meta-analysis[33] that examined the prevalence of *H pylori* in gastroesophageal reflux disease, apart from BE, and found a significant lower prevalence of *H pylori* infection among patients with gastroesophageal reflux than among those without the disease. All the above could mean that *H pylori* infection might be protective against gastroesophageal reflux disease and its neoplastic complications, ie, BE and thereby AC.

The mechanism by which *H pylori* might protect against gastroesophageal reflux, BE, and finally esophageal AC might be related to modulation of gastric acidity. It has been shown that corpus gastritis is associated with lower gastric output in both the basal and stimulated conditions.[34] Furthermore, the corpus inflammation, induced by the *H pylori cagA*–positive strain,[35] accelerates the progression to multifocal atrophic gastritis, with destruction of gastric glands,[42] leading to further loss of gastric acidity. Nonetheless, whatever the mechanism, our results have to be interpreted carefully because the clinical relevance of a lower prevalence of *H pylori* in patients with BE and AC is unclear.

In AC subgroup meta-analyses, no significant heterogeneity was noted in both *H pylori* and the *H pylori cagA*–positive strain prevalence. However, significant heterogeneity was noted in BE. For this reason, sensitivity analyses were performed to exclude any possible influence of a single study. It was found that the removal of 1 study[32] had a significant effect on the pooled ORs in both the *H pylori* and the *H pylori cagA*–positive strain meta-analyses, with restoration of homogeneity after its removal. This was a study from Spain with an *H pylori* prevalence in patients that was significantly higher than that found in controls (87.3% vs 74.6%, respectively; $P = .013$). However, despite the significant heterogeneity found, we included this study in our meta-analysis, because heterogeneity is expected in a meta-analysis of epidemiologic data.[41,42]

It has been suggested[25] that *H pylori* infection, through induction of gastric atrophy and subsequent reduced gastric acidity, might result in overgrowth of bacteria, which produce nitrosamines and thereby increase esophageal SCC risk. However, in our meta-analysis we did not find any significant association between *H pylori* or *H pylori cagA*–positive strain prevalence and SCC because there were studies reporting inverse,[26,38] positive,[27] or no association.[33] A significant heterogeneity was noted in the subgroup meta-analyses concerning the relationship of SCC prevalence with *H pylori* and the *H pylori cagA*–positive strain, and for this reason, sensitivity analyses were performed to exclude any possible influence of a single study. We did not find any significant effect on the pooled ORs; therefore, the observed significant heterogeneity could be attributed to other factors concerning methodology or biology rather than the influence of a single study. Such factors could include sex, age, geographic location, smoking, alcohol consumption, and time from serum collection to the diagnosis.

In conclusion, the results showed a statistically significant inverse relationship between the prevalence of *H pylori* as well as the *H pylori cagA*–positive strain with both BE and AC, whereas no significant relationship was shown for SCC. The significant inverse relationship between *H pylori* and both BE and AC could suggest that *H pylori* infection protects from these entities. However, because relatively few studies are available so far and the current evidence is yet limited, the necessity to conduct large-scale prospective studies with an adequate methodologic quality and proper controlling for possible confounders to obtain valid results should be emphasized.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org.

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A preliminary report of some of the results in this study has been presented at the XIX International Workshop on *Helicobacter* and Related Bacteria in Chronic Digestive Inflammation, Wroclaw, Poland, September 7-9, 2006.

Table 1. The Main Characteristics of Studies, Selected for Meta-Analysis, Examining the *H pylori* Prevalence in Patients and Controls

	H pylori prevalence (*H pylori* positive/total number)						
	AC		SCC		BE		
Study	Patients	Controls	Patients	Controls	Patients	Controls	Type of study
Newton,[19] 1997, United States	NA	NA	NA	NA	4/16	9/25	Cohort study
Chow,[20] 1998, United States	38/129	86/223	NA	NA	NA	NA	Retrospective case-control study; population-based controls
Vicari,[21] 1998, United States	7/21	26/57	NA	NA	15/48	26/57	Cohort study
Rugge,[22] 2000, Italy	NA	NA	NA	NA	19/53	36/53	Retrospective, case-control study
Lord,[23] 2000, Australia	6/35	67/214	NA	NA	15/91	67/214	Cohort study
Vieth,[24] 2000, Germany	66/138	468/712	NA	NA	562/1054	468/712	Cohort study
Weston,[25] 2000 United States	5/34	96/217	NA	NA	90/255	96/217	Cohort study
Siman,[26] 2001, Sweden	10/44	67/149	NA	NA	NA	NA	Nested case-control study
El-Omar,[27] 2003, United States	35/108	84/212	32/53	84/212	NA	NA	Population-based case-control study
Wu,[28] 2003, United States	49/80	230/356	NA	NA	NA	NA	Population-based case-control study
Ye,[29] 2004, Sweden	18/97	198/499	32/85	198/499	NA	NA	Population-based case-control study
De Martel,[30] 2005, United States	19/51	76/149	NA	NA	NA	NA	Nested case-control study
Wu,[31] 2005, Taiwan	NA	NA	28/127	74/171	NA	NA	Case-control study
Ferrandez,[32] 2006, Spain	NA	NA	NA	NA	91/104	159/213	Cohort study
Kamangar,[33] 2007, China	NA	NA	254/335	727/992	NA	NA	Cohort study
Σ (*H pylori* positive)/Σ (total) (%)	253/737 (34.3%)	1398/2788 (50.14%)	346/600 (57.6%)	1083/1874 (57.8%)	796/1621 (49.1%)	961/149 (57.7%)	

AC, adenocarcinoma; SCC, squamous cell carcinoma; BE, Barrett's esophagus; NA, not available.

Table 2. The Main Characteristics of Studies, Selected for Meta-Analysis, Examining the *H pylori cagA* (+) Strain Prevalence in Patients and Controls

	H pylori cagA (+) prevalence (*cag A* (+) *H pylori*/total number)						
	AC		SCC		BE		
Study	Patients	Controls	Patients	Controls	Patients	Controls	Type of study
Chow,[20] 1998 United States	12/38	46/86	NA	NA	NA	NA	Retrospective case-control study; population-based controls
Vicari,[21] 1998 United States	0/7	11/26	NA	NA	2/15	11/26	Cohort study
Rugge,[22] 2000, Italy	NA	NA	NA	NA	6/19	26/36	Retrospective, case-control study
Veezi,[24] 2000 United States	NA	NA	NA	NA	2/83	11/60	Cohort study
Wu,[26] 2003, United States	15/80	87/356	NA	NA	NA	NA	Population-based case-control study
Ye,[29] 2004 Sweden	42/97	293/499	63/85	293/499	NA	NA	Population-based case-control study
Kudo,[25] 2004, Colombia	NA	NA	NA	NA	8/23	19/24	Cohort study
De Martel,[30] 2005, United States	9/51	44/149	NA	NA	NA	NA	Nested case-control study
Jansson,[36] 2005 Sweden	42/189	293/820	63/167	293/820	NA	NA	Population-based case-control study
Ferrandez,[32] 2006, Spain	NA	NA	NA	NA	67/104	116/213	Cohort study
Kamangar,[33] 2007, China	NA	NA	178/335	552/992	NA	NA	Cohort study
Σ (*H pylori cag* positive)/Σ (total) (%)	120/462 (26%)	774/1936 (40%)	304/587 (51.7%)	1138/2311 (49.2%)	87/244 (35.6%)	185/359 (51.5%)	

AC, adenocarcinoma; SCC, squamous cell carcinoma; BE, Barrett's esophagus; NA, not available.

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Helicobacter pylori and Non-malignant Diseases

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Keywords
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Abstract

In recent years, the focus of *Helicobacter pylori* clinical research has been mainly on gastric malignancy. However, the role of *H. pylori* in non-malignant diseases, such as peptic ulcer, gastroesophageal reflux disease (GERD) and non-ulcer dyspepsia, as well as non-steroidal anti-inflammatory drug consumption, is still of great interest. A 1- to 2-week course of *H. pylori* eradication therapy is an effective treatment for *H. pylori*-positive peptic ulcer disease and a positive *CagA* status is a predictor for successful eradication of *H. pylori*. Antral prostaglandin-E2-basal levels appear to be critical for the development of aspirin-induced gastric damage in subjects without *H. pylori* infection. In clinical practice, among patients treated with proton-pump inhibitors, *H. pylori* status has no effect on the speed or degree of GERD symptom relief. For the management of dyspepsia in primary care, antisecretory therapy confers a small insignificant benefit compared to strategies based on *H. pylori* testing while these latter strategies may be cost-effective. *H. pylori* eradication therapy has a small but statistically significant effect on *H. pylori*-positive non-ulcer dyspepsia. An economic model suggests that this modest benefit may still be cost-effective but more research is needed.

Peptic Ulcer Disease

Over the last year a number of papers concerning various aspects of the association of *Helicobacter pylori* with peptic ulcer disease (PUD) have been published. The majority examined the effectiveness of various *H. pylori* treatments and will be reviewed in the Treatment section.

Murakami et al. [1] examined the possible relationship between peptic ulcer recurrence and the presence or absence of maintenance therapy with an H_2-receptor antagonist administered until evaluation of *H. pylori* eradication. The results of this study suggested that maintenance therapy with an H_2-receptor antagonist post-eradication therapy is likely to greatly reduce the ulcer recurrence rate without affecting the evaluation of *H. pylori* eradication.

Eradication of *H. pylori* reduces the relapse rate of PUD. Ford et al. examined the magnitude of this effect in their systematic review and meta-analysis [2], which was an update of a previous systematic review [Cochrane Database Sits Rev. 2004;(4):CD003840]. The primary outcomes were an increase in the proportion of peptic ulcers healed initially and an increase in the proportion of patients free from relapse following successful healing. Eradication therapy was compared to placebo or pharmacologic therapies in *H. pylori*-positive patients. Secondary aims included symptom relief and adverse events. Sixty-three trials were eligible and 56 trials were finally included. For duodenal ulcer healing, eradication therapy was superior to ulcer-healing drugs (34 trials, 3910 patients, relative risk [RR] of ulcer persistence = 0.66; 95% confidence interval [CI] 0.58–0.76) and no treatment (two trials, 207 patients, RR 0.37; 95% CI 0.26–0.53). For gastric ulcer healing, no significant difference was detected between eradication therapy and ulcer-healing drugs (14 trials, 1572 patients, RR 1.25; 95% CI 0.88–1.76). In preventing duodenal ulcer recurrence, no significant differences were detected between eradication therapy and maintenance therapy with ulcer-healing drugs (four trials, 319 patients, RR of ulcer recurrence = 0.73; 95% CI 0.42–1.25), but eradication therapy was superior to no treatment (27 trials, 2589 patients, RR 0.20; 95% CI 0.15–0.26). In preventing

 Helicobacter 12 (Suppl. 1): 20–30

gastric ulcer recurrence, eradication therapy was superior to no treatment (11 trials, 1104 patients, RR 0.29; 95% CI 0.20–0.42). The authors concluded that a 1- to 2-week course of *H. pylori* eradication therapy is an effective treatment for *H. pylori*-positive PUD. The role of the *cag*A status of *H. pylori* strains as a predictive factor for the outcome of eradication therapy is controversial. Suzuki et al. in their systematic review and meta-analysis [3] confirmed the importance of the presence of *cag*A as a predictor for successful eradication of *H. pylori*.

Non-Steroidal Anti-inflammatory Drug Consumption

Last year relatively few studies examined the association of *H. pylori* with non-steroidal anti-inflammatory drugs (NSAIDs). The mechanisms by which *H. pylori* and low-dose aspirin induce gastric damage are not completely elucidated. Thus, Venerito et al. [4] evaluated the effects of low-dose aspirin on gastric damage, mucosal prostaglandin-E2 levels, and cyclooxygenase-enzyme expression in relation to *H. pylori* status. They concluded that in healthy subjects, low-dose aspirin given for 1 week does not affect cyclooxygenase expression or mucosal prostaglandin-E2 levels. Antral prostaglandin-E2 basal levels appear to be critical for development of aspirin-induced gastric damage in subjects without *H. pylori* infection.

Gastroesophageal Reflux Disease

As with the association of *H. pylori* infection and NSAIDs, last year few studies were devoted to the association of *H. pylori* and gastroesophageal reflux disease (GERD). Several studies suggested that proton-pump inhibitors suppress gastric acid more effectively in *H. pylori*-infected than in non-infected patients, but no evaluation of the short-term clinical response was performed. De Boer et al. [5] studied whether *H. pylori* infection influences the response rate or speed of symptom control in patients with GERD treated with rabeprazole. They did not find an effect on either of these parameters according to *H. pylori* status. Infected patients and non-infected patients can therefore be treated with a similar dose of rabeprazole. When treating heartburn with rabeprazole, physicians do not need to consider the patients' *H. pylori* status and most patients (> 80%) have adequate symptom relief after just a few days of treatment. Rabeprazole (10 mg b.i.d.) is often administered as an eradication therapy for *H. pylori* and has also been proposed as a therapy for refractory GERD. However, there has not been a comprehensive assessment of its acid-suppressive effects. Shimatani et al. [6] compared the acid-suppressive effects of rabeprazole (10 mg b.i.d. or 20 mg b.i.d.). They found that the effects of the two rabeprazole doses were the same in *H. pylori*-positive patients, whereas in *H. pylori*-negative subjects, 20 mg b.i.d. was superior for prevention of nocturnal acid breakthrough.

The effect of *H. pylori* eradication on the development of GERD is controversial. Vakil et al. [7] determined the incidence of symptoms of reflux disease and erosive esophagitis and also their relationship to changes in histologic gastritis in patients with non-ulcer dyspepsia (NUD) over 12 months. Gastric biopsies were scored using the modified Sydney classification. The results showed that antrum-predominant gastritis is the most common pattern of gastritis seen in NUD in Western populations. Heartburn and regurgitation improve after eradication therapy or placebo in patients with NUD and the development of esophagitis is uncommon.

The impact of long-term acid suppression on the gastric mucosa remains controversial. Lundell et al. [8] reported on further observations concerning an established cohort of patients with GERD, after a 7-year follow up. Among the original cohort randomized for either omeprazole treatment or anti-reflux surgery, 117 and 98 patients remained in the medical and surgical arms, respectively. Gastric biopsies were taken at baseline and throughout the study. Results showed that long-term omeprazole therapy does not alter the exocrine oxyntic mucosal morphology in *H. pylori*-negative patients, but mucosal endocrine cells appear to be under proliferative stimulation; changes in mucosal inflammation and atrophy were observed in *H. pylori*-positive patients.

Dyspepsia and Non-ulcer Dyspepsia

Hu et al. [9] compared empirical prokinetics, the *H. pylori* test-and-treat strategy and empirical endoscopy in a 1-year study on primary-care patients presenting with dyspepsia. They found the three strategies equally effective. Empirical prokinetic treatment was the least expensive but peptic ulcers were sometimes missed, whereas the *H. pylori* test-and-treat strategy was indeed the most cost-effective option. An economic evaluation of empirical antisecretory therapy versus *H. pylori* test-and-treat strategy in the management of dyspepsia patients presenting in primary care was performed by Jarbol et al. [10]. Thus, a randomized trial in 106 general practices in the County of Funen, Denmark, was designed in order to obtain clinical outcome measures and resource utilization data prospectively. Seven hundred and twenty-two dyspeptic patients presenting with more than 2 weeks of epigastric pain or discomfort were randomized in one of three initial management strategies: 1, empirical antisecretory therapy; 2, testing for *H. pylori*, or 3, empirical antisecretory therapy, followed by *H. pylori* testing if symptoms improved.

Cost-effectiveness and incremental cost-effectiveness ratios of the strategies were determined. They concluded that empirical antisecretory therapy confers a small but not significant benefit and costs more than test-and-treat strategies for *H. pylori*, therefore it is probably not a cost-effective strategy for the management of dyspepsia in primary care. Undoubtedly, *H. pylori* is the main cause of PUD but its role in NUD is less clear. Moayyedi et al. examined this question in their recent systematic review and meta-analysis [11] which was an update of a previous systematic review [Cochrane Database Syst Rev. 2005;(1):CD002096]. They determined the effect of *H. pylori* eradication on dyspepsia symptoms in patients with NUD. They included all parallel group randomized controlled trials (RCTs) comparing drugs to eradicate *H. pylori* with placebo or other drugs known not to eradicate *H. pylori* for patients with NUD and 21 RCTs met the inclusion criteria. Eighteen trials compared antisecretory dual or triple therapy with placebo antibiotics with or without antisecretory therapy, and evaluated dyspepsia at 3–12 months. Seventeen of these trials gave results as dichotomous outcomes evaluating 3566 patients and there was no significant heterogeneity between the studies. There was a 10% relative risk reduction in the *H. pylori* eradication group (95% CI 6–14) compared to the placebo. The number needed to treat in order to cure one dyspeptic patient was 14 (95% CI 10–25). Three further trials compared bismuth-based *H. pylori* eradication with an alternative pharmacologic agent. These trials were smaller and had a shorter follow up but suggested that *H. pylori* eradication was more effective than either H_2-receptor antagonists or sucralfate in treating NUD. *H. pylori* eradication therapy has a small but statistically significant effect in *H. pylori*-positive NUD patients. An economic model suggests that this modest benefit is cost-effective but requires confirmation.

Conflicts of interest

The authors have declared no conflicts of interest.

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GASTROENTEROLOGY

Incidence and etiology of acute non-malignant upper gastrointestinal bleeding in northern Greece

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aspirin, gastrointestinal bleeding, *Helicobacter pylori*, non-steroidal anti-inflammatory drugs, peptic ulcer.

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Abstract

Aim: To evaluate the incidence and etiology of acute non-malignant upper gastrointestinal bleeding (ANMUGIB) in northern Greece due to increased use of non-steroidal anti-inflammatory drugs (NSAIDs), including low-dose aspirin (L-A), exposure and geographical variability of *Helicobacter pylori* (Hp) seroprevalence.

Methods: A retrospective study of 110 patients admitted for hematemesis or melena during a 6-month period. All patients had undergone a gastrointestinal (GI) endoscopy during hospitalization. The presence of Hp was identified by biopsies and a ^{13}C-urea breath test in the case of Hp(−) biopsy bleeding peptic ulcer (BPU). The activity of ANMUGIB was assessed according to Forrest's classification. Statistical analysis was made by the χ^2-test and Yates' correction.

Results: Most patients were in the two medium age groups with no significant difference between them ($P < 0.001$). NSAID or L-A (100 mg/day) use was reported in 42.73% of patients in a ratio 1:1 ($P > 0.1$) and Hp infection was found in 29.09% of patients. BPU, with approximately two-thirds in the bulb, erosions and varices were the most frequent sources. Hp infection was found in 60.65% of BPU, 65.57% were related to NSAIDs or L-A and 8.19% were non-Hp non-NSAID/L-A BPU. Flat spots were most commonly found with a significant difference ($P < 0.001$) to other stigmata of recent bleeding, except for clean base.

Conclusions: In northern Greece, persons aged over 40 years are prone to ANMUGIB with a non-significant relationship to males. Hp infection and medication use, such as NSAIDS and L-A, are deeply involved in its etiology. Non-Hp non-NSAID/L-A BPU are a small proportion. ANMUGIB seems to have a generally good prognosis.

Introduction

Acute upper gastrointestinal (GI) bleeding is a very frequent medical emergency. Although it is most commonly of benign pathology, it is associated with considerable morbidity and mortality rates up to 13%, as well as the financial cost of health services. Several changes have influenced the epidemiological pattern of acute non-malignant upper gastrointestinal bleeding (ANMUGIB) during the last decades.[1]

The recognition of the role of *Helicobacter pylori* (Hp) infection in ulcer formation was followed by the development of eradication therapies with a consequent reduction in ulcer recurrence and rebleeding. Further conservative management progress, characterized by the introduction of proton pump inhibitors (PPI) with strong anti-suppressive properties, has been combined with advances in diagnostic and therapeutic endoscopic practices.[1] Thus, the incidence of peptic ulcer disease and ulcer complications has shown a marked decline with an increase observed in bleeding peptic ulcer (BPU), especially in elderly patients.[2]

A global demographic trend towards an aged society with comorbidities and greater medication consumption is occurring.[3] In addition to the extensive worldwide use of non-steroidal anti-inflammatory drugs (NSAIDs), whose adverse effects are principally of gastrointestinal origin, an increased use of anti-platelet agents for cardiovascular protection, mainly low-dose aspirin (L-A), appears to be an emerging risk factor for ANMUGIB.[4,5] Hp seroprevalence, which is much higher in developing countries and even higher among subgroups within many regions, varies by geographic location, ethnic background, socioeconomic conditions and age.[6]

The frequency of Hp infection and NSAID or L-A intake in promoting ANMUGIB in a population is therefore associated with its Hp prevalence and drug exposure. Various studies of the

incidence, etiology and characteristics of ANMUGIB can be found in the English literature but there is a relative lack of relevant data concerning Greek patients.

Methods

The aim of the present study was to quantify the incidence and to evaluate the etiology of ANMUGIB in Greek patients. Data from all patients who had been admitted for hematemesis or melena in a university hospital in northern Greece over a 6-month period were retrospectively collected from medical and nursing reports. Patients with a history of previous gastric surgery and those who had been diagnosed with gastric malignancy were excluded. An upper GI endoscopy had been performed in all patients during hospitalization. The presence of Hp had been initially identified by biopsies taken from both the antrum and corpus of the stomach (rapid urease test and/or histology). Patients with Hp-negative BPU were re-evaluated by a ^{13}C-urea breath test 2 weeks after treatment cessation. The activity of ANMUGIB had been assessed according to Forrest's classification as follows: Forrest I active bleeding: IIa non-bleeding visible vessel (NBVV); IIb adherent blood clot; IIc flat spots; III clean ulcer base. Information collected included age, gender, recent intake of NSAIDs and L-A, endoscopic findings, histological findings of Hp infection, ^{13}C-urea breath test results, hemoglobin (Hb) levels at admission, units of blood transfused, and length of hospital stay.

Statistical analysis was performed using the paired χ^2-test followed by Yates' correction. A P-value less than 0.05 was considered to be statistically significant.

Results

ANMUGIB accounted for 110/151 (72.85%) of acute non-malignant gastrointestinal bleeding events with 58/110 (52.73%) cases of ANMUGIB occurring in males and 52/110 (47.27%) in females. Mean age of patients was 59.73 years ± 15.42 years. The two medium age groups included the majority with 50 (33.11%) and 58 (38.41%) patients being divided in the groups of 41–65 years and 66–80 years, respectively ($P < 0.001$). Only 12 patients (7.95%) were aged over 81 years (Table 1). According to medical history, 47 (42.73%) patients were on NSAIDs, including L-A (100 mg/day), treatment. Gender distribution showed no significant difference in NSAID-related ANMUGIB ($P > 0.1$) with 23 (48.94%) patients being males and 24 (51.06%) being females. The drug ratio was 1:1 ($P > 0.1$) with 24 (21.82%) patients being treated with NSAIDs other than L-A and 23 (20.91%) with L-A alone, respectively (Fig. 1). Based on histology, the presence of Hp was identified in 32/110 (29.09%) patients with ANMUGIB.

Table 1 Age distribution of acute non-malignant upper gastrointestinal bleeding

Patient age group (years)	Acute non-malignant upper gastrointestinal bleeding (%)
16–40	19 (17.27)
41–65	40 (36.36)
66–80	44 (40.1)
>81	7 (6.36)

BPU was the major source of ANMUGIB in 61/110 (55.45%) patients. A bulbar location was found in 39 (63.93%) and a gastric location in 22 (36.07%) patients, respectively. Gastroduodenal erosions in 19/110 (17.27%) and varices in 16/110 (14.55%) patients were the second and third most frequent sources, respectively. A slightly increased incidence of BPU was observed in favor of 33/61 (54.1%) males whereas gastroduodenal erosions occurred in 12/19 (63.16%) females. BPU was the predominant source of ANMUGIB in 40/47 (85.11%) patients on NSAID or L-A treatment with a bulbar location in 24 (51.06%) and a gastric location in 16 (34.04%) patients. Gastroduodenal erosions in 6/47 (12.76%) patients were the second source of NSAID-related ANMUGIB (Table 2).

The presence of Hp infection was found in 37/61 (60.65%) BPU. More specifically, 32/61 (52.46%) Hp(+) BPU were diagnosed by histology and five by a ^{13}C-urea breath test performed in the remaining 29 patients after PPI cessation. Recent intake of NSAIDs or L-A was recorded in 40/61 (65.57%) BPU. Twenty-three out of 40 (57.50%) patients were also Hp(+) with 21/40 (52.5%) being identified by histology. Non-Hp non-NSAID/L-A BPU were found in 5/61 (8.19%) patients (Fig. 2). According to Forrest's classification, flat spots were the most common stage of recent bleeding in 23 out of 61 (37.7%) BPU. They had a statistically significant difference ($P < 0.001$) in comparison to the others, except for ulcer clean base (17/61, 27.87%). Adherent blood clot (9/61, 14.75%) was the third most common endoscopic finding, followed by active bleeding (7/61, 11.48%) and NBVV (5/61, 8.2%), respectively (Fig. 3). Mean Hb levels of patients at admission and red cell transfusion units required during

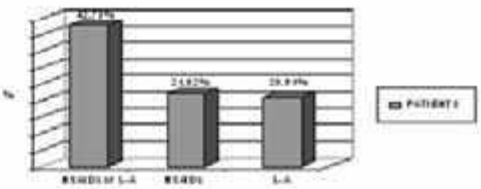

Figure 1 Non-steroidal anti-inflammatory drugs (NSAIDs) and low-dose aspirin (L-A) intake in acute non-malignant upper gastrointestinal bleeding.

Table 2 Sources of acute non-malignant upper gastrointestinal bleeding and gender distribution of these

Source of bleeding	Patients (%)	Males (%)	Females (%)
Peptic ulcer	61 (55.45)	33 (54.1)	28 (45.9)
Bulbar ulcer	39 (35.45)	23 (58.97)	16 (41.03)
Gastric ulcer	22 (20)	10 (45.45)	12 (54.55)
Erosions	19 (17.27)	7 (36.84)	12 (63.16)
Varices	16 (14.55)	10 (62.5)	6 (37.5)
Esophagitis	7 (6.36)	4 (57.14)	3 (42.86)
Mallory Weiss	6 (5.46)	4 (66.67)	2 (33.33)
Angiodysplasia	1 (0.91)	0 (0)	1 (100)

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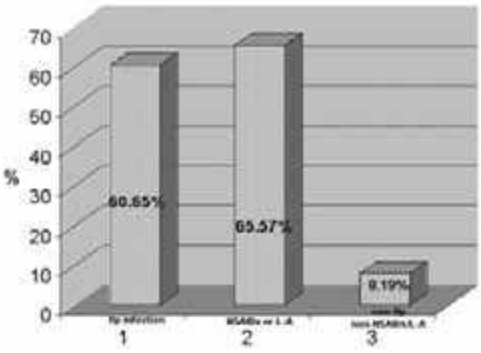

Figure 2 Major etiological factors of bleeding peptic ulcers (BPU).

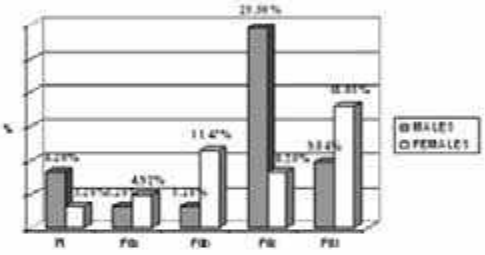

Figure 3 Gender distribution of stigmata of recent bleeding according to Forrest's classification.

hospitalization were 10.28 g/dL and 1.785 g/dL, respectively. The average length of stay in hospital was 7.375 days.

Discussion

The majority of acute upper GI bleeding events are of benign pathology. Their management involves emergency room physicians, gastroenterologists, endoscopists and other health care personnel.

In a generally aging population, evolving changes in etiological factors and therapeutic advances have affected the natural history of ANMUGIB. Gastric Hp infection, the second most common infectious disease in humans, is generally known to be a major etiological factor in peptic ulcer disease, which in turn is a major source of ANMUGIB.[7] However, Hp seroprevalence, which increases with age, has decreased over the last decades in industrialized countries, demonstrating variance from country to country.[8] An association of NSAIDs with ANMUGIB also occurs due to prostaglandin depletion.[9,10] Although the probability that any given individual user of NSAIDs will suffer an ANMUGIB is fairly low, widespread patient exposure to NSAIDs can translate into a major health burden.[11] The increasing use of long-term L-A treatment for the prevention of cardiovascular and cerebrovascular disease significantly reduces gastric mucosa prostaglandin levels.[12]

The absence of a statistically significant difference in gender distribution, in favor of males, was not in accord with a previous Greek study.[13] This might be attributed to the increasing exposure of females to NSAIDs or L-A, as shown in the Nurses' Health study's results which reported NSAID use ≤1 day by 42% of females aged over 51 years and L-A use ≥6 days weekly by approximately 25% of females.[14,15]

The mean age of patients was 59.73 years, being higher than that of a study from the bordering country Turkey.[16] Aged between 41 years and 81 years had a significant difference compared to other groups ($P < 0.001$), with a slightly higher proportion between 66 years and 80 years. An issue worth mentioning here is that approximately 90% of Americans aged over 65 years report NSAID use at least once weekly and 34% take them on a daily basis.[14] The highest age group (over 81 years) was small in size, which is mainly attributed to possible death before reaching hospital due to comorbidities.[1]

Approximately half of the patients with ANMUGIB had used NSAIDs, including L-A. A higher proportion of NSAID-related cases and only a slightly higher one were reported in a previous Greek study and a south Greek study, respectively.[17,18] Gender distribution of drug-related ANMUGIB had no significant difference ($P > 0.1$), such as ANMUGIB in total, but was in favor of females.[19]

Approximately one-fifth of patients were receiving L-A and another one-fifth were receiving NSAIDs other than L-A. In a recent English study, 27.1% and 13.5% of patients had reported a recent intake of L-A and NSAIDs other than L-A, respectively.[20]

Histological findings suggested the presence of Hp infection in approximately one-third of ANMUGIB cases. Our results were in accord with those of the study just mentioned but significantly lower compared to the Greek study.[17,20] As generally reported in the literature, BPU was the major source of ANMUGIB in half of the patients.[21] An American study, however, has estimated its frequency to be one-third of ANMUGIB patients.[22] The ratio of the duodenal BPU to the gastric BPU was 2:1, similar to the Greek study mentioned earlier.[13] Gastroduodenal erosions and varices were the second and third most frequent bleeding lesions, respectively. Both a study from the Netherlands and the USA have supported the same order whereas the opposite has been shown in a previous Greek and French study.[1,13,21,23] A non-significant relationship ($P > 0.1$) of BPU to males in contrast to the predominance of females in gastroduodenal erosions was observed.

The vast majority of NSAID-related ANMUGIB cases were a consequence of BPU, as seen in other studies, but the prevalence of bulbar location was in contrast to many previous studies.[22,24,25] However, it was supported by another Greek study.[26]

An increase has been noticed in the proportion of BPU not attributed to Hp infection.[6] Sixty percent of BPU cases infected by Hp are in accord with a very recent study's results.[27] A study from our neighboring Turkey reported a rate of 66.7%.[28] A mean infection rate of 79.8% was, however, calculated from 32 studies, ranging between 46% and 100%.[29] According to more recent literature, it ranges between 57% and 73%.[30] The geographic, ethnic and socioeconomic variability of Hp seroprevalence, and the dif-

ferent grade of histological reliability, which is correlated with the number and site of biopsies taken from the stomach due to patchy distribution of Hp or prior antibiotic treatment use, might be possible explanations for this discrepancy.[6,17,28] Despite the high specificity of histology, it has also been shown that prompt initiation of PPI treatment may have a negative impact on the accuracy of almost all Hp diagnostic methods in patients with BPU during hospitalization. Serology, the only exception, appears to be a rather inaccurate test owing to quite variable results and a need for previous validation in each geographic region to be considered reliable.[31] The positive results of a ^{13}C-urea breath test, performed 2 weeks after PPI cessation, in Hp(−) BPU had no significant difference when compared to the histological findings. As regards to the lower Hp prevalence of BPU than that of uncomplicated peptic ulcers, this might reflect a failing incidence of Hp ulcers due to bleeding or perhaps a different pathophysiological mechanism.[29]

Almost two-thirds of BPU were associated with a recent intake of NSAIDs including L-A, whereas the study from Turkey reported the association in to be 79.2%.[28] More than half of NSAID ulcers were infected by Hp while a recent study found two-thirds of them to be Hp(+).[32] However, the role of concomitant Hp infection in NSAID ulcers still remains controversial.[10,33]

Non-Hp non-NSAID/L-A BPU accounted for 8.19% of cases in contrast to double this in the study of Hung *et al*. Gastric acid hypersecretion, bile reflux, and infection by other Hp species have also been proposed.[29]

According to Forrest's classification, flat spots (IIc), observed in approximately one-third of BPU, had a significant difference from other stigmata of recent bleeding ($P < 0.001$), with the exception of ulcer clean base (III). In contrast, the most frequent endoscopic stage was III and only a small proportion of BPU was on IIc in an American study.[22] The next most common findings were adherent blood clot, active bleeding and NBVV, which was the same order as that reported in another recent study.[34] However, the lower proportion of active bleeding compared to that study might be explained by a limited number of early endoscopies (within 24 h). Stigmata of recent bleeding are, actually, more likely to be found if endoscopy is performed within 12–18 h after admission.[35] For example, most ulcers with NBVV are reported to evolve into clean-based ulcers within 2 days.[22]

Our patients presented with slightly higher mean initial Hb levels than those in a study from Hong Kong and a slightly lower mean transfusion number compared to another study, whereas the average length of stay was slightly higher than that reported in an American study.[24,35,36]

Taking into consideration the limitations of our study of patients admitted to a university hospital in northern Greece, the results indicate that persons aged over 40 years are prone to ANMUGIB. A non-significant relationship of ANMUGIB to males and an NSAID-related association to females was apparent. A deep involvement of certain medication use and Hp infection in the etiology of ANMUGIB, and especially BPU, occurred. In particular, NSAIDs as well as L-A can induce BPU. A rather small proportion of BPU, however, can be attributed neither to Hp nor to prior NSAID/L-A exposure. Re-evaluation of Hp negative BPU patients with a ^{13}C-urea breath test 2 weeks after PPI cessation seems not only to be reliable but is also a simple and easily performed supplement diagnostic option for Hp infection. In general, the prognosis of ANMUGIB seems to be good on the basis of mean Hb levels at admission, red cell transfusions, activity of bleeding and length of hospital stay, irrespective of etiology. Large trials are, however, required for a better understanding of the etiological factors of ANMUGIB among different geographic areas in the 21st century so that effective preventive strategies can be scheduled.

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Primary MALT lymphomas of the stomach: A pathological study of 18 cases

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RESUMEN

Objetivo: es difícil que alguien que padezca un linfoma gástrico de tipo MALT pueda librarse de la enfermedad... a menos que se le trate con medicación para *Helicobacter pylori*.

Material y métodos: se analizó una cohorte de 18 pacientes. Diez huéspedes tenían linfoma gástrico de tipo MALT y se trataron con resección gástrica como tratamiento inicial. Ocho recibieron antibióticos frente a *Helicobacter pylori* como tratamiento inicial. En los 18 pacientes se evaluaron la presencia de *Helicobacter pylori*, los hallazgos endoscópicos y los rasgos patológicos. Se realizó una inmunohistoquímica para valorar el bcl-2 y el p53.

Resultados: los pacientes con linfoma MALT de grado bajo: a) dieron positivo a *Helicobacter pylori* (5 de 5), b) tenían una lesión superficial (5 de 5), c) no tenían afectados los ganglios linfáticos (5 de 5), y d) se estadificaron a la baja por comparación con los pacientes con tumores de grado alto. El bcl-2 fue positivo en 4 de los 5 tumores de grado bajo y el p53 fue positivo en 12 de 13 de los de grado alto. El estudio de los pacientes durante un seguimiento de 5 años (n = 18) reveló que todos los tumores menos uno de grado bajo siguieron siendo superficiales sin progresión. Estos tumores eran bcl-2+/p53-, mientras que el único con inmunofenotipo bcl-2+/p53+ progresó hasta convertirse en un tumor de bajo grado ulcerado tras la desaparición de *Helicobacter pylori*. Se observó una regresión completa en 6 de los 8 pacientes del grupo no tratado con cirugía (n = 8) tras la erradicación de *Helicobacter pylori*. Estos tumores eran superficiales, de bajo grado, con ganglios negativos y bcl-2+/p53 no concluyente (n = 2); superficiales, de bajo grado, con ganglios negativos y bcl-2+/p53- (n = 2), y ulcerativos, de grado alto, con ganglios negativos y bcl-2+/p53- (n = 2). Los dos tumores persistentes eran ulcerativos, de grado alto con ganglios negativos y bcl-2+/p53+.

Conclusión: el linfoma gástrico de tipo MALT, *Helicobacter pylori*-positivo, superficial, de grado bajo y bcl-2+/p53- desaparece tras la erradicación de *Helicobacter pylori*.

Palabras clave: Linfoma gástrico MALT. Bcl2 oncogenes. p53 oncogenes. Inmunohistoquímica.

ABSTRACT

Aim: it is doubtful that whoever is suffering from gastric MALT lymphoma will escape from the disease, if treated with medication against helicobacter pylori.

Material and methods: a cohort of 18 patients was analysed. Ten hosts had primary gastric malt lymphoma and were treated with gastric resection as the initial therapy. Eight hosts received antibiotics against *Helicobacter pylori* as the initial treatment. In all 18 patients *Helicobacter pylori* status, endoscopic findings and pathology features were evaluated. Immunohistochemistry was performed to assess the bcl-2 and p53 status.

Results: patients with low grade MALT lymphoma: a) were *Helicobacter pylori* positive (5 of 5), b) had a superficial lesion (5 of 5), c) had no lymph node involvement (5 of 5), and d) were downstaged by comparison to patients with high grade tumor. Bcl-2 was positive in 4 of 5 low grade tumors, and p53 was positive in 12 of 13 high grade ones. Investigation of patients with 5-year follow up (n = 18) revealed that all but one low-grade tumors remained superficial with no progression. These tumors were bcl-2+/p53-, and the one with a bcl-2+/p53+ immunophenotype progressed to an ulcerated low-grade tumor after disappearance of *Helicobacter pylori*. Complete regression was found in 6 of 8 patients from the non surgically treated group (n = 8) after *Helicobacter pylori* eradication. These tumors were superficial/low grade/node negative/bcl-2+/p53 inconclusive (n = 2), superficial/low grade/node negative/bcl-2+/p53- (n = 2), and ulcerative/high grade/node negative/bcl-2+/p53- (n = 2). The two persistent tumors were ulcerative/high grade/node negative/bcl-2+/p53+.

Conclusion: gastric MALT lymphoma *Helicobacter pylori*+/superficial/low grade/bcl-2+/p53- will disappear after *Helicobacter pylori* eradication.

Key words: Gastric MALT lymphoma. Bcl2 oncogene. p53 oncogene. Immunohistochemistry.

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INTRODUCTION

The gastrointestinal tract is the most common site of the primary extranodal lymphomas and gastric lym-

phomas are featured in the majority of the cases (1). However gastric lymphoma accounts for only 1-10% of all gastric malignant neoplasms (2).

Since the first publication of Isaacson and Wright (3), the concept of low grade (LG) B-cell lymphoma of mucoca-associated lymphoid tissue (MALT) seems to have become widely accepted and has been incorporated into the new classification scheme (REAL) as extranodal marginal zone B-cell lymphoma (4). In addition, transformation to high grade (HG) lymphomas from LG MALT lesions has been described by many authors (2,5-7).

Acquired MALT accumulates in the stomach after *Helicobacter pylori* (*Hp*) infection, and *Hp* can be found in most of the cases. More recently, eradication of *Hp* has been added to the potentially effective stomach-conserving therapies for LG-MALT-lymphomas (8-12).

Compared with node-based lymphomas, these MALT lymphomas of the stomach are recognized as a distinct disease entity with a characteristic presentation, histological spectrum and clinical behaviour (13). They differ in at least three instances: a) in localized stages, they behave as focal tumors and may, therefore, be curable by radical resection; b) most tumors of LG malignancy have a distinct morphology; and c) relapse of the disease may occur exclusively within the gastrointestinal tract, even long after remission (5).

The over expression of p53 protein has been recently reported in HG gastric lymphomas (2,14). An inverse relationship between bcl-2 protein expression and p53 expression in primary gastric lymphomas has been reported and bcl-2 positivity was found to decrease whereas p53 positivity increased significantly as the histologic grade advances (2).

Although gastric lymphomas are not rare in our country, there are few detailed studies in this subject. The aim of this retrospective study is to reinvestigate primary gastric lymphomas and characterize them histopathologically and immunophenotypically. The expression of bcl-2 oncoprotein and p53 oncogene were evaluated in the same cases, using immunohistochemical methods.

MATERIAL AND METHODS

During the period from 1990 to 2000 ten patients with primary malt lymphoma were retrospectively analysed and underwent gastric resection as initial treatment. Eight patients with malt lymphoma who received antibiotics against *Helicobacter pylori (Hp)* were also included in the study. All 8 patients were positive for *Hp* and were investigated prospectively. Writen informed consent was obtained from each subject. In all 18 patients the presence of *Hp*, endoscopic findings, pathologic findings of the biopsy and reseceted specimen, and immunohistochemical expression of bcl-2, p53, CD3, and CD20 antigens were evaluated. *Hp* infection was diagnosed by biopsy, the urease test, histology, and culture in patients receiving antibiotics, and was diagnosed histologically in patients who underwent surgery. Treatment included omeprazole 20 mg and amoxicillin 1.5 g twice a day plus clarithromycin 800 mg twice a day, for two weeks.

Histologic sections prepared from paraffin blocks were routinely stained with hematoxylin-eosin (HE) and examined through a standard light microscope. The cases were divided into 4 groups according to the MALT lymphoma concept (15). The histologic features of MALT lymphoma proposed by Issacson are as follows: low grade lymphoma shows proliferation of centrocyte-like cells that occasionally invade the glands (lymphoepithelial lesions) and have a marked tendency toward plasma cell differentiation. The presence of lymphoid follicles in or around the tumor is a constant finding. In high grade MALT lymphoma, large, transformed lymphoid cells show diffuse proliferation with or without areas of low grade MALT lymphoma (16). The lymphoid infiltration in gastric biopsy specimens were classified according to the criteria of Wotherspoon et al. (11). To assess changes on repeat biopsy, the confidence in a diagnosis of lymphoma was expressed on a scale of 0-5 (15). Patients were staged by physical and ORL examinations, blood tests, ultrasonography, whole-body computed tomography (CT) scanning, and endoscopic ultrasonography of the gastric lesion, according to modified Ann Arbor classification criteria (17-20).

Expression of Bcl-2 protein (Bcl-2, DAKO), p53 protein (D0-7, DAKO), CD3 (DAKO), and CD20 (DAKO), was also assessed in paraffin embedded sections. For immunoperoxidase staining of Bcl-2, p53, CD3, and CD20, we applied the microwave oven heating technique, which has been shown to be effective for the retrieval of masked epitopes. The findings were classified as follows: (-), no reactive cells; (+), scattered positive cells; (2+), nests of positive cells; (3+), diffuse positive cells.

The antibiotic-treated group was then prospectively followed with regular endoscopic biopsy, and each response was histologically evaluated and graded using the histologic scoring system proposed by Wotherspoon et al. (11): with a posttreatment score < 3 evidencing lymphoma regression, a score of 3 indicating partial response, and a score of 4-5 indicating no response. Follow-up was carried out every 3-6 months, with clinical evaluation; upper endoscopy plus multiple biopsies for histologic, bacteriologic, and immunohistochemical studies; and endoscopic ultrasonography (EUM 2 or 3, Olympus).

Modified Giemsa (MG) staining was performed using a mixture of 60 ml distilled water and 2.6 ml MG solution. After the treatment of sections with this solution, they were left overnight and then treated with 1% acetic acid. An immunohistochemical study was performed using a Labeled Streptavidin Biotin (LSAB) Universal kit (DAKO) according to the instructions of the manufacturer and 3-amino, 9-ethyl-carbazole (AEC) was used as chromogen. The primary antibodies used are prediluted forms of anti-bcl-2 protein, p53 oncoprotein, CD20, and CD3 antibodies. Five-

micron thick paraffin sections were left overnight in 37 °C in autoclave for overnight. After deparaffinization for 7 minutes, they were treated with 800 KW antigen retrival. The immunostained slides were examined by light microscopy. The staining was considered positive where more than 10% of the lymphoma and neoplastic plasma cells were stained strongly. When less than 10% of the cells were stained positively, it was scored as weak positivity.

Data were evaluated with the chi-square or the Fisher exact test. A p value < 0.05 was considered statistically significant.

RESULTS

Of the 10 patients with primary malt lymphoma who were surgically treated, 1 was classified as harboring low grade tumor and 9 as harboring high grade tumors (low > high:2, low < high:4, high:3 patients; low > high describes low grade B-cell lymphomas of MALT with small areas of high grade lymphoma and low < high describes high grade B-cell lymphomas with small areas of low grade components of MALT, high describes pure high grade lymphomas). The age of the patients with high grade components ranged from 34 to 81 years (median, 60.2 years), and the male-to-female ratio was 9:5. The correlations between the histologic type of lymphoma and other factors, such as the presence of *Hp*, macroscopic tumor type, depth of invasion, lymph node involvement, clinical stage, and expression of Bcl-2 (Fig. 1), and p53 (Fig. 2), are shown in tables I and II.

All 10 patients were *Hp* positive, the one low grade tumor was superficial. All the high grade tumors were ulcerated, no lymph node involvement by any tumor was present. All patients were staged as EI, the one low grade tumor was bcl-2 positive and p53 negative, and all the high grade tumors were bcl-2 +/p53+.

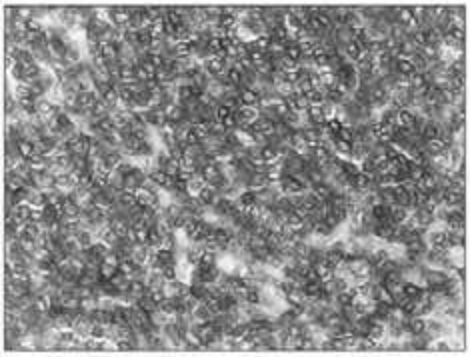

Fig. 1. MALT-type gastric lymphoma. Tissue section. Bcl-2 expression. Immunostain X 200.

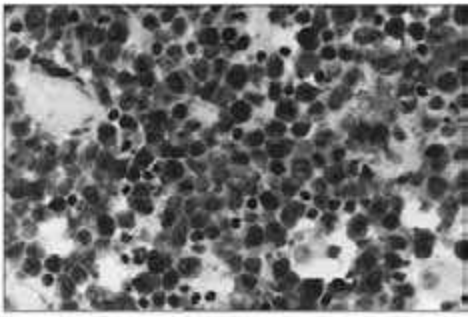

Fig. 2. MALT-type gastric lymphoma. Tissue section. p53 expression. Immunostain X 200.

Of the 8 patients harboring MALT lymphoma who were treated with antibiotics, 4 were classified in the low grade tumor category and 4 in the high grade category (low > high:1, low < high:2, high:1 patient). All were *Hp* positive; all low grade tumors were superficial, all high grade tumors were ulcerated; no lymph node involvement was present by the CT-scanning; all patients were staged as EI; one low grade tumor was bcl-2 negative; three high grade tumors were p53 positive; the one low grade/bcl-2 negative tumor was positive by p53 immunostaining; two low grade tumors were bcl-2 inconclusive/p53 positive).

Table I. Characteristics of patients who were treated with antibiotics against *Hp*

Case	Macroscopic features	Grade	Nodes	bcl-2	p53
1	Superficial	Low	0	-	-
2	Superficial	Low	0	+/-	+
3	Superficial	Low	0	+/-	+
4	Superficial	Low	0	-	+
5	Ulcerative	High	0	+	+
6	Ulcerative	High	0	+	+
7	Ulcerative	High	0	+	+
8	Ulcerative	High	0	+	+

Table II. Characteristics of patients who were treated with surgery

Case	Macroscopic features	Grade	Nodes	bcl-2	p53
1	Superficial	Low	0	+	-
2	Ulcerative	High	0	+	+
3	Ulcerative	High	0	+	+
4	Ulcerative	High	0	+	+
5	Ulcerative	High	0	+	+
6	Ulcerative	High	0	+	+
7	Ulcerative	High	0	+	+
8	Ulcerative	High	0	+	+
9	Ulcerative	High	0	+	+
10	Ulcerative	High	0	+	+

All 18 tumors were CD20 positive/ CD3 negative.

A 5-year follow-up of all 18 subjects disclosed that all low grade tumors except one, remained superficial with no progression and these tumors were bcl-2+/p53; the one exception from the rule was positive by both antigens immunostaining. This patient developed an ulcerated low grade lesion after eradication of *Hp*.

Complete regression was found in 6 out of 8 not surgically treated patients, after *Hp* infection was no more present: these tumors were superficial/low grade/node negative/bcl-2+/p53 inconclusive (n = 2), and superficial/low grade/node negative/bcl-2+/p53- (n = 4). The remaining two were ulcerative/high grade/node negative/bcl-2 +/p53+.

There was a statistically significant difference between p53 positivity between low grade and high grade cases ($p = 0.0302$ for overall positivity, $p = 0.0036$ for only strong positivity). In high grade cases positivity of bcl2 and p53 oncogens were compared, p53 expression was found higher than bcl2 expression and the difference between the positivity of these oncogens in high grade cases, was statistically significant ($p = 0.0422$). There was no statistically significant difference between their expression in low grade tumors.

DISCUSSION

Gastric lymphomas account for the majority of the extranodal lymphomas and until the 1980s, all of the NHL classifications were unavailable for gastrointestinal lymphomas (2). A new classification for these lymphomas according to LG MALT concept, was proposed by Isaacson et al. in 1983 (3) and since then, this classification has become widely used. Later, transformation from LG lesions to HG lymphoma was described by some authors (2,5,21). In addition, others described mixed grade (LG B-cell lymphoma of MALT with a focal HG component) (22). HG gastric MALT lymphomas were reported to be more frequent than LG lesions (5,19,23). In Nakamura's study, among 233 cases, 43% of the cases were HG, 30% were LG, 12% were LG with focal HG, 6% other B, 6% other T-cell lymphomas (9). In Chan's series, 12 of 48 cases were LG, 26 were HG and 10 were mixed LG and HG (5). However, His et al. found LG MALT lymphomas more (22 of 48 cases) (6). By contrast, some investigators reported that not MALT type, but the diffuse large cell type of lymphoma, was highest in incidence among primary gastric lymphomas in Japan (24).

In the study of Nakamura, among macroscopic types, most of tumors that appeared as mass forming type proved to be HG tumors (2). As the MALT accumulates in stomach after *Hp* infection, *Hp* can be found in most of the lymphoma cases (10,11). In our study *Hp* was positive in all 18 cases (100%).

The vast majority of primary gastric lymphomas are of B-cell origin. T-cell tumors are very rare (2,22,24). In our series all lymphomas demonstrated a B-cell immunophenotype as they were stained positive with CD20.

The bcl-2 proto-oncogene, which was cloned from the break-point region of t(14;18) chromosomal translocation is frequently observed in the follicular lymphoma and the expression of bcl-2 protein has been detected in various nodal lymphomas. However, few articles have evaluated the expression of bcl-2 protein by immunohistochemical technique in primary lymphomas. The frequency of bcl-2 positivity in these studies is different according to grade (2,7). In the largest series, bcl-2 protein expression was detectable in 68% of primary gastric lymphoma cases.

The overexpression of p53 protein either with or without a gene mutation has been reported in various tumors including nodal lymphomas. However, immunohistochemical analysis of p53 expression in gastric lymphomas has been done in a few studies (2,14).

In conclusion, primary gastric lymphomas comprise a group of distinctive clinicopathologic entities. Most of LG B-cell gastric lymphomas are of MALT type and appear to arise in MALT aquired as a reaction to *Hp* infection. LG MALT NHL may undergo HG transformation, and LG component can be shown in HG MALT lesions. There is an inverse correlation between the expression of bcl-2 and p53 proteins in gastric lymphomas. p53 oncoprotein positivity increases where bcl-2 oncoprotein positivity decreases as the histologic grade advances. This result suggests that the expression of bcl-2 and p53 may be associated with a transition from LG to HG tumors.

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Helicobacter pylori infection as an environmental risk factor for migraine without aura

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Abstract Helicobacter pylori (*H. pylori*) infection has recently been associated with various extraintestinal pathologies and migraine. The aim of this study was to investigate the correlation of the *H. pylori* infection with the pathogenesis of migraine without aura, especially in cases not affected by endogenous risk factors, like hereditary pattern or hormonal fluctuations.
A total of 49 outpatients (37 females and 12 males; age range: 19–47 years; mean age: 31.±14 years) affected by migraine without aura was evaluated. We divided them in 2 subgroups: a) with positive familial history, and/or with menstrual type of migraine b) with negative familial history and with menstrual unrelated type of migraine. *H. pylori* infection was diagnosed by the 13 C- urea breath test (INFAI – test).
Control subjects consisted of 51 patients without any primary headache history (38 females; mean age of 32.±14.4 years; range 21–49 years), who underwent upper gastrointestinal (GI) endoscopy for investigation of anaemia or non ulcer dyspepsia. *H. pylori* detection was based on the histologic analysis of gastric mucosa biopsy.
The prevalence of *H. pylori* infection was significantly higher in the migraineurs without aura compared to controls (p=0.016).
The prevalence of *H. pylori* infection was significantly high in the mixed and in the female group of our patients without other predisposing factors for migraine without aura (81 and 87% respectively), while in the same groups with predisposing factors (menstruation and/or family history) the prevalence was only 36 and 37% respectively (p=0,001 for the first group and p=0,002 for the second group).
Our results seem to highlight the role of *H. pylori* infection as a probable independent environmental risk factor for migraine without aura, especially in patients that are not genetically or hormonally susceptible to migraine.

Keywords Migraine without aura • *Helicobacter pylori* infection • Hereditary patterns • Menstrual migraine

Introduction

Occurrence of migraine has been associated with clinically obvious or subclinical extracranial infection (herpes labialis, pharyngitis, cystitis, vaginitis, mycosis and gastrointestinal inflammation) [1, 2]. It has also been demonstrated that *Helicobacter pylori* infection has a possible role in precipitation of migraine [3–6], while other data support a simple co-occurrence of *H. pylori* infection and migraine [7–9].

H. pylori infection is the most common cause of gastri-

tis and gastric and duodenal ulcers. The association of *H. pylori* infection and various extraintestinal pathologies, such as coronary heart disease, primary Raynaud phenomenon, migraine, Alzheimer's disease and mild cognitive impairment, has recently been addressed [3–6,10–13].

Migraine without aura seems to be caused by a combination of genetic and environmental factors, whereas migraine with aura is probably determined largely by genetic factors [14, 15].

The aim of this study was to investigate the correlation of the *H. pylori* infection with the pathogenesis of migraine without aura, especially in cases not affected by endogenous risk factors, like hereditary pattern or hormonal fluctuations.

Materials and methods

Study design

A total of 49 outpatients (37 females and 12 males; age range: 19–47 years; mean age: 31.6±14 years) affected by migraine without aura were evaluated. The cases came from a series of consecutive patients referred to the Neurological Department of the General Hospital of Athens 'Laiko' from January to December 2003. Our study was non-randomised and enrolled the above-mentioned consecutive patients. All patients gave their consent prior to the inclusion in the study and no one refused to participate.

In addition to personal data, participants were asked detailed questions regarding their type of headache: the duration, the frequency, the location, the quality and intensity, the average number of headache attacks per month or per year, as well as the occurrence of nausea, vomiting, photophobia, phonophobia or osmophobia and the occurrence and duration of associated neurological symptoms. Data about relationship with menstruation and hereditary patterns were collected. The lifetime history of migraine for every patient was obtained using the Diagnostic Interview for Headache Syndromes (DIHS), a structured instrument which was developed to collect the major subtypes of headache as defined according to the 2004 International Headache Society (IHS) Criteria for Headache Syndromes [16]. All patients underwent brain CT or MRI without any pathological findings. In all cases *H. pylori* infection was diagnosed by the INFAI test, a non-invasive, simple, highly sensitive and specific method to assess *H. pylori* infection [5].

Migraine was classified as migraine with or without aura according to IHS criteria [9] and as menstrual migraine according to MacGregor's definition, as migraine attacks exclusively starting on the first day of menstruation ±2 days and at no other time of the menstrual cycle [17].

Complete pedigrees on first-degree relatives including offspring over 18 years old were obtained for the family of every one of our migraineurs. Face-to-face or telephone assessments of first-degree relatives, including parents, siblings and offspring >18 years were conducted by clinically experienced neurologists who were blind to the diagnostic status of the proband. Information on relatives who were deceased or refused to participate was obtained from multiple relatives. Because the intention was to include non-interviewed relatives, it was not possible to discriminate between specific subtypes of migraine (with and without aura). Final diagnostic assessments were made by a panel of experienced clinicians based on all available information.

The familial history was considered as positive if any of the first-degree relatives had a headache history estimated as migraine and was considered as negative if the headache history of all the first-degree relatives were either negative or indicative of another type of primary or secondary headache [14, 15].

The total population of our migraine outpatients was divided into cases without aura and cases with aura. We selected only patients without aura (duration: 1–20 years, median: 9) and we divided them in 2 subgroups: (a) with positive familial history, and/or with menstrual type of migraine and(b) with negative familial history and with menstrual unrelated type of migraine.

Control subjects consisted of 51 patients without migraine (38 females; mean age of 32.8±14.4 years; range 21–49 years), who underwent upper gastrointestinal (GI) endoscopy for investigation of anaemia or non-ulcer dyspepsia at the Department of Gastroenterology and Pathophysiology of the University of Athens during the last year, but in whom endoscopy did not reveal any obvious finding. Mean age and gender ratios did not differ between migraineurs and control participants. *H. pylori* detection was based on the histologic analysis of gastric mucosa biopsy. Face-to-face or telephone assessments of headache history of control subjects were conducted by clinically experienced neurologists who were blind to the biopsy result. Only subjects without any primary headache history were accepted.

Our study was performed in accordance with the Declaration of Helsinki and was approved by the appropriate investigational review board, which is the ethics committee of the Medical School of the University of Athens.

Statistics

Statistically significant differences between groups were assessed using the χ^2 test and Yates correction when appropriate. Statistical significance was accepted as $p<0.05$.

Results

The INFAI test was positive in 61% of the total population of our patients (30 out of 49 migraineurs were infected by *H. pylori*). *H. pylori* bacteria were histologically present in 37.25% of our control subjects (19 out of 51 subjects were infected). The prevalence of *H. pylori* infection was significantly higher in the migraineurs ($p=0.016$, odds ratio: 2.65,

95% CI: 1.18–5.96).

The prevalence of *H. pylori* infection in the total group of patients (males and females), was significantly higher in migrainous patients with negative familial history and negative correlation with menstruation (81% *vs.* 36%, p=0.001, odds ratio: 7.70, 95% CI: 2.09–28.33) than migrainous patients with positive familial history and/or menstrual migraine (22 out of 27 patients *vs.* 8 out of 22 patients were infected) (Figure 1).

Similar findings were observed in the subgroup of female patients, where the prevalence of *H. pylori* infection was respectively 86% (18 out of 21 women with menstrual unrelated migraine and/or negative familial history were infected) and 37% (6 out of 16 women with menstrual migraine and/or positive family history were infected) (p=0.002, odds ratio: 10.00, 95% CI: 2.04–48.80) (Figure 2).

We also found a higher prevalence of the infection in male patients with negative family history. Among 6 men without family history, 4 were infected, whereas among 6 men with family history, 2 were infected (p_{exact}=0.564).

Discussion

The prevalence of *H. pylori* infection was significantly higher in the migraineurs without aura compared to controls (p=0.016).

Compared with migraineurs with menstrual migraine and/or positive family history, migraineurs with menstrual unrelated migraine and negative family history were significantly more frequently infected by *H. pylori*.

The prevalence of *H. pylori* infection was significantly high in the mixed and in the female group of our patients without other predisposing factors for migraine without aura (81 and 87% respectively), while in the same groups with predisposing factors (menstruation and/or family history) the prevalence was only 36 and 37% respectively. The difference was statistically significant for both the above-mentioned groups (p=0.001 for the first group and p=0.002 for the second group).

The prevalence of infection was also higher in male patients with negative family history, but the size of this

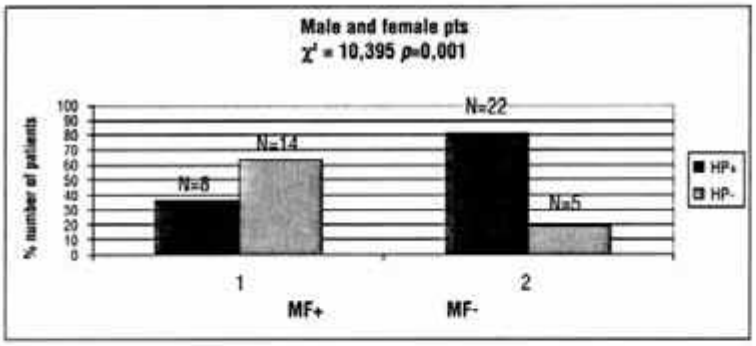

Fig. 1 The prevalence of *H. pylori* infection in the total group of patients. *HP+*, *H. pylori* infected; *HP–*, *H. pylori* not infected; *MF+*, patients with menstrual migraine and/or familial history; *MF–*, patients without menstrual migraine or familial history

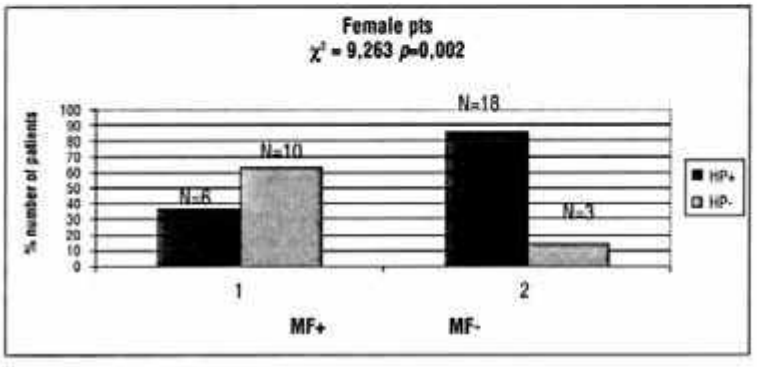

Fig. 2 The prevalence of *H. pylori* infection in the subgroup of female patients. *HP+*, *H. pylori* infected; *HP–*, *H. pylori* not infected; *MF+*, patients with menstrual migraine and/or familial history; *MF–*, patients without menstrual migraine or familial history

subgroup was small.

These results are in agreement with literature data showing higher frequency of *H. pylori* infection in migraineurs [3–5] and considering that *H. pylori* should be examined in migrainous patients as a possible risk factor that can contribute to clinical improvement of duration and intensity of migraine attacks with its eradication. Consequently, they are in disagreement with other data supporting a simple co-occurrence of *H. pylori* infection and migraine [6–9].

We selected this subgroup of migraineurs (without aura), because this subtype is more often connected with menstrual migraine [18] and we were wondering if there are other significant triggering factors in non-menstrual migraine without aura. But the most important reason for selecting this migraine subgroup was the fact that migraine without aura seems to be caused by a combination of genetic and environmental factors whereas migraine with aura is probably determined largely or exclusively by genetic factors [14, 15]. A previous study reported that spouses to probands with migraine without aura had a slightly increased risk of migraine without aura, while spouses to probands with migraine with aura had no increased risk of migraine with aura [15]. According to our results it is possible that the responsible environmental factor may be *H. pylori* infection.

A significantly higher prevalence of a specific type of *H. pylori*, the CagA-positive *H. pylori* strains, has been demonstrated only in migraine with aura patients [19]. A plausible mechanism underlying the association between CagA-positive *H. pylori* strains and vascular diseases may be linked to the significantly more intense chronic immuno-inflammatory response that follows colonisation of the gastric mucosa by these strains; cytokines (in particular, interleukin-1, -6, -8 and interferon-γ) and other molecules endowed with proinflammatory, vasospastic and proalgogen properties conceivably may induce a systemic vasculopathy within various arterial districts [10]. We suppose that a similar mechanism produces the association between *H. pylori* infection and migraine without aura but it was impossible to assess the CagA status of *H. pylori* in our patients in that period of time in our hospital, so we cannot conclude anything about this specific topic (CagA-positive strains and migraine without aura). Other recent results do not support the role of oxidative stress in patients suffering from *H. pylori* infection and migraine [20].

Our findings are based on a small sample size and cannot produce consistent results. But we think that they can be useful to clinicians in order to test the possibility of *H. pylori* infection in migraineurs without aura and to researchers as a trigger for larger studies.

Taking into account that hereditary pattern [14, 15] and hormonal fluctuations [18] are known and well established endogenous risk factors for migraine, our results seem to highlight the role of *H. pylori* infection as a probable independent environmental risk factor for migraine without aura, especially in patients that are not genetically or hormonally susceptible to migraine. We suggest that these cases should be more intensively examined for this infection and probably could have the best improvement with its eradication.

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Letter to the Editor

Helicobacter pylori and multiple sclerosis

Keywords: multiple sclerosis; *Helicobacter pylori*; molecular mimicry; apoptosis; neurodegenerative diseases

We read with considerable interest the paper by Li et al. (2007) on the inverse association between *Helicobacter pylori* (*Hp*) and multiple sclerosis (MS) based on serological test. Their discussed data, however, suggest that a clean environment is associated with conventional MS and, on the other hand, an infectious one is associated with opticospinal MS. The latter is characterized by a higher age at onset and other features, thereby considering having a distinct immune mechanism; it is associated with a pronounced shift in the responses of T helper 1 (Th1) and T cytotoxic 1 (CT1) cells and a distinct HLA haplotype (Kira, 2003).

The early events underlying MS remain uncertain, although several environmental factors may be involved. In this respect, the possibility that microorganisms can cause MS has recently been addressed; notably Epstein–Barr virus, human herpes virus 6 and *Chlamydia pneumoniae* are under investigation (Giovannoni et al., 2006). These pathogens by eliciting inflammation may cause neurological damage resulting in MS.

Likewise, *Hp* infection (*Hp*-I) has been implicated in extradigestive vascular conditions caused by vascular dysregulation, frequently detected in MS and other relative neurodegenerative diseases including glaucoma, Alzheimer's disease (AD) and mild cognitive impairment (MCI) (Kountouras et al., 2004, 2006, in press). In this regard, using histology, recognized as the practical gold standard for the diagnosis of current *Hp*-I, our pilot study showed a strong association between *Hp*-I and MS (Table 1). Although serological test establishes the presence of *Hp*-I, it does not discriminate between current and old infections. Such a distinction is crucial 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 (Kountouras et al., 2005), thereby contributing and possibly perpetuating neural tissue damage. Moreover, eradicating *Hp*-I might alter MS pathophysiology.

Besides, we previously demonstrated an association between *Hp*-I and glaucoma and AD or MCI (Kountouras et al., 2004, 2006, in press). In AD, brain infiltration by pathogens acts as a trigger or co-factor for AD, with *Herpes simplex* virus type 1 and *Chlamydophila* being implicated most frequently, as also suggested for MS (Giovannoni et al., 2006). Furthermore, *Hp*-I is more frequent in Guillain–Barré syndrome (GBS) which may also share common pathogenetic mechanisms with MS, anti-*Hp* titer might reflect advanced GBS clinical status, and these antibodies are also associated with involvement of the proximal parts of peripheral nerves in GBS patients (Kountouras et al., 2005). The infecting organism induces humoral and cellular immune responses that, because of the sharing of the mentioned homologous epitopes (molecular mimicry), cross-react particularly with ganglioside surface components of peripheral nerves. Immune reactions against target epitopes in Schwann cell surface membrane or myelin result in GBS (Kountouras et al., 2005). Interestingly, molecular mimicry of host structures by the saccharide portion of lipopolysaccharides of the gastrointestinal pathogens *Campylobacter jejuni* and *Hp* are thought to be connected with the development of autoimmune sequelae observed in GBS (Kountouras et al., 2005). It has been reported that the target molecules of the specific antibody against *Hp* VacA in the cerebrospinal fluid of GBS patients are probably associated with some components of the peripheral nerve myelin, thereby suggesting a potential role in the immune responses of demyelinating GBS paticnts (Kountouras et al., 2005); the sequence homology found between *Hp* VacA and human Na^+/K^+–ATPase A subunit suggests that antibodies to VacA target ion channels in abaxonal Schwann cell plasmalemma resulting in demyelination in some patients. In this respect, some data also suggest a pathogenic antibody response to native myelin oligodendrocyte glycoprotein reported in MS patients (Zhou et al., 2006). Therefore, abnormalities of humoral immunity appear to play a role in the pathogenesis of these neurodegenerative diseases including MS.

Furthermore, glial activation and expression of cytokines may act in synergy with other genetic and acquired environmental risks culminating in the development of these immune-mediated diseases involving defective immune regulation and autoimmunity (Kountouras et al., 2007). Specifically, microglia as well as astrocytes, macrophages and dendritic cells [(DCs), the most potent antigen-presenting cells (APCs)] are the immune effector cells in the central nervous system concomitantly with inflammatory brain disease and play a significant role in the host defense against invading agents including microorganisms. MHC class II (HLA-DR) antigen-positive reactive microglia were observed in AD or MS (Huitinga et al., 2004), and enriched microglial cultures alone can stimulate TL responses or the $CD4^+$ TL subset (Kountouras et al., 2007). 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

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Table 1
Helicobacter pylori positivity in patients with multiple sclerosis (MS) and anemic controls

Characteristics	MS patients ($n=29$)	Anemic controls ($n=25$)	Odds ratio (95% CI)	P value
Age, mean (SD), years	38.65 (8.94)	44.44 (13.8)	–	0.066
Women, *n* (%)	16 (55.17)	11 (44)	–	0.413
Positive urease test (gastric mucosa), *n* (%)	21 (72.41)	10 (40)	3.428 (1.057–11.332)	0.016
Serum anti-*H. pylori* IgG concentration, mean (SD), U/mL	48.82 (37.93)	17.51 (13.11)	–	<0.001
Histologically confirmed presence of *H. pylori*, *n* (%)	24 (82.75)	12 (48)	3.9 (1.102–13.8925)	0.007

neurodegenerative diseases including MS; secretion of substantial levels of pro-inflammatory Th1-type cytokine TNF-α leads to TNF-α-related apoptotic neuronal cell death in these diseases including MS (Buntinx et al., 2004). Notably, apoptotic, rather than necrotic, microglia-associated nerve cell death appears as likely to underlie a number of common neurological conditions including AD, Parkinson's disease, glaucoma (ocular AD) and MS (Kountouras et al., 2007). The latter disease, for example, is also crucially dependent on the activation of pro-inflammatory Th1Ls by APCs, resistance of TLs 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 the disease (Town et al., 2005; Okuda et al., 2006).

Summarizing, the abovementioned data describe the current evidence for cellular immune defective and apoptotic mechanisms playing an important role in the neurodegenerative process in MS and other relative diseases.

As in the case of MS, comparable cellular immune-mediated and apoptotic pathogenic features can also be introduced for *Hp*-I. This bacterium elicits a complex immune response, thereby initiating innate and adaptive immune responses. The dense infiltration of the gastric mucosa with cells of the immune system suggests that a complex interplay between APCs and other immune cells may be important for the development of *Hp*-induced gastric pathologies (Kountouras et al., 2007). *Hp*-I up-regulates the expression of MHC class II antigens on gastric epithelium; gastric epithelial cells may acquire APC properties in *Hp*-I by de novo expression of HLA-DR and costimulatory molecules. Moreover, *Hp* induces DC activation, maturation as well as antigen presentation; its outer membrane proteins are capable of activating DCs and DCs pulsed with *Hp* were shown to induce Th1 effector responses. Therefore, *Hp*-associated gastroduodenal pathologies can be regarded as a Th1-driven immunopathological response to a number of *Hp* antigens. Specifically in *Hp*-related autoimmune gastritis, cytolytic TLs infiltrating the gastric mucosa cross-recognize different epitopes of *Hp* proteins and gastric H^+–K^+–ATPase autoantigens (a significant proportion of the $CD4^+$ T cell clones proliferated in response to H^+–K^+–ATPase showing a Th1 profile), and this bacterium may lead to gastric autoimmunity via molecular mimicry (Kountouras et al., 2007); activation of gastric H^+–K^+–ATPase-specific Th1 TLs is critical in the pathogenesis of gastric autoimmunity and atrophy in humans. A predominant *Hp*-specific Th1 response characterized by high TNF-α, interferon (IFN)-γ, interleukin (IL)-2 and IL-12 production leading to gastric epithelial cell apoptotic damage. Several studies reported that the Fas/Fas ligand (Fas-L) system is involved in *Hp*-induced apoptosis and T-cell-mediated cytotoxicity via Fas/Fas-L signaling may contribute to the induction of apoptosis in gastric epithelial cells during *Hp*-I (Kountouras et al., 2007). Inflammatory cytokines present during *Hp*-I, such as IFN-γ, enhance the activation of the Fas-signaling pathway in vitro. In this regard, Fas and TNF-α-receptor type 1 (TNF-R1) expressed on gastric epithelial cells from *Hp*-infected patients are responsible for the accelerated cell apoptosis; TNF-α induces apoptotic death of gastric parietal cells contributing to the atrophy and hypochlorhydria of the gastric mucosa in chronic *Hp*-I. Additional evidence indicates that *Hp* is capable of inducing apoptotic effects through the mitochondrial apoptotic pathway involving activation of the proapoptotic proteins Bax and Bak, activation of certain caspases or through inducible nitric oxide (NO); NO is a rapidly diffusing gas and a potent neurotoxin that may contribute to the apoptotic neuronal cell death in degenerative neuropathies including AD (Fernandez-Vizarra et al., 2004), glaucomatous optic neuropathy (Kountouras et al., 2007) and MS (Bojc, 2004). Therefore, there is evidence for the irregular cellular immune and apoptotic mechanisms playing an important role in the *Hp*-associated gastrointestinal pathologies and potentially affecting the neurodegenerative process in MS.

The abovementioned data do not establish causality, because apart from the other Koch and Hill's causation criteria, this requires showing that eradication of *Hp*-I alters the course of MS and the other relative neurodegenerative diseases. However *Hp*-I may influence the pathophysiology of MS by (1) promoting platelet aggregation and activation also proposed to play pathophysiologic roles in MS (Kountouras et al., 2004, 2006); (2) releasing proinflammatory and vasoactive substances such as cytokines (IL-1,-6,-8,-10,-12, TNF-α, IFN-γ) or eicosanoids (leukotrienes, prostaglandins catalyzed by cyclo-oxygenase enzymes) involved in a number of vascular disorders including MS and other MS-related neuropathies such as AD, glaucoma, Parkinson disease or GBS (Kountouras et al., 2004, 2006; Dhib-Jalbut et al., 2006); (3) stimulating mononuclear cells to produce a tissue factor-like procoagulant that converts fibrinogen into fibrin (Kountouras et al., 2004, 2006); (4) causing the development of cross-mimicry between endothelial and *Hp* antigens; (5) producing reactive oxygen metabolites and circulating lipid peroxides also involved in the pathophysiology of MS (Kountouras et al., 2004, 2006; Dhib-Jalbut et al., 2006); and (6) influencing the apoptotic process, an important form of cell death in many neurodegenerative diseases including MS (Kountouras et al., 2004, 2006; Dhib-Jalbut et al., 2006).

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The American Journal of Clinical Nutrition

Letters to the Editor

Helicobacter pylori may be involved in cognitive impairment and dementia development through induction of atrophic gastritis, vitamin B-12–folate deficiency, and hyperhomocysteinemia sequence

Dear Sir:

We read with considerable interest the article by Haan et al (1), which concluded that homocysteine (Hcy) is an independent risk factor for both dementia and cognitive impairment without dementia (CIND) in a cohort of Latin Americans residing in California and, moreover, higher plasma vitamin B-12 concentrations may reduce the risk of Hcy-associated dementia or CIND.

The background prevalence of *Helicobacter pylori* serum positivity in this population (Hispanic Mexicans) is ≈60% (2, 3). In particular, 79% of Hispanic volunteers residing in Los Angeles were shown to harbor *H. pylori* in gastric biopsy samples and all had histologic gastritis (4).

In this respect, although degenerative diseases of the central nervous system (CNS), including Alzheimer disease (AD), have an increasingly greater effect in elderly populations, their association with *H. pylori* infection has not been thoroughly researched. This issue was recently addressed in 2 studies (5, 6). A higher seropositivity for anti-*H. pylori* immunoglobulin (Ig) G antibodies was reported in AD patients than in age-matched controls (5). However, this serologic test has limitations because it does not discriminate between current and old infections (7). Such a distinction is essential because current *H. pylori* infection induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves (7) and thereby affect or perpetuate neural tissue damage. Moreover, eradication of *H. pylori* infection might delay AD progression, particularly at early disease stages. On the basis of histologic analysis of gastric mucosa biopsy samples for the documentation of *H. pylori* infection, we investigated whether *H. pylori* infection is associated with AD by introducing the histologic method that is established as the actual gold standard for diagnosis of *H. pylori* infection (7). In our cohort of Greek patients, 88% of the AD patients had histologically proven *H. pylori* infection, whereas the rate of infection was significantly lower in the anemic control group (46.7%) (6). Moreover, histologic multifocal chronic gastritis (body and antrum atrophy) was observed in the vast majority of our patients as compared with controls (6, 7). These patterns of *H. pylori*–related chronic gastritis have also been reported by others (7). Importantly, an increased serum Hcy concentration has been shown in our AD patients (7). Chronic gastritis, as a result of *H. pylori* infection, can lead to malabsorption of vitamins (B-12) and folate, which results in the failure of methylation by 5-methyl-tetrahydrofolic acid and, hence, in the accumulation of Hcy (7). Elevated Hcy, in turn, could trigger endothelial damage and result in atherothrombotic disorders and AD. In this respect, investigators reported that *H. pylori*–induced chronic atrophic gastritis or atrophic gastritis per se decreases serum vitamin B-12 and folate concentrations, thereby increasing Hcy—a potent contributor to vascular disorders; serum Hcy concentrations correlated inversely with serum vitamin B-12 and folate concentrations and positively with atrophic scores (7). Hcy appears to be an independent risk factor not only for dementia and AD, as mentioned by Haan et al, but also for vascular disease. It is thought to be implicated in endothelial damage and neurodegeneration via oxidative injury in these diseases (7); oxidative damage has also been described in the brain of subjects with mild cognitive impairment (MCI), which suggests that oxidative damage may be one of the earliest events in the onset and progression of AD. It has been shown that the serum Hcy concentration correlates with the severity of dementia, and it is a significant predictor of the severity of dementia (7). From another vantage point, *H. pylori* infection is actually associated with vitamin B-12 deficiency or iron deficiency anemia, whereas eradication of *H. pylori* infection is associated with the reversal of vitamin B-12 deficiency or of iron deficiency and an improvement in anemia (8).

Extending our findings, we investigated 63 consecutive patients with amnestic MCI, and 35 anemic controls who underwent upper gastrointestinal endoscopy and histologic and serologic examinations (9). The prevalence of *H. pylori* infection was 88.9% in MCI patients and 48.6% in controls, as confirmed by biopsy ($P < 0.001$; odds ratio: 8.47; 95% CI: 3.03, 23.67). Mean serum anti-*H. pylori* IgG concentration and plasma total Hcy titer were also higher in MCI patients than in controls. When compared with the anemic participants, MCI patients had histologic multifocal (body and antral) gastritis more often. Interestingly, the positivity status for *H. pylori* serology appeared to correlate with cognitive deterioration in our *H. pylori*–positive MCI patients (9).

Considering the abovementioned data, we speculate that *H. pylori* infection might contribute, at least in part, to the pathogenesis of MCI and AD through induction of chronic atrophic gastritis, vitamin B-12–folate deficiency, and Hcy sequence. It would be of interest to know whether Haan et al took into account comparable data from participants in the Sacramento Area Latino Study on Aging (SALSA), who would be expected to have a high prevalence of *H. pylori* infection. Such data appear to be crucial in shedding light on *H. pylori* infection, which may influence the pathophysiology of the MCI-AD sequence by: *1*) promoting platelet and platelet-leukocyte aggregation, also proposed to play pathophysiologic roles in AD development (7, 9); *2*) releasing proinflammatory and vasoactive substances involved in a number of vascular disorders, including MCI, AD, and other AD-related neuropathies such as glaucoma, defined as "ocular AD" (7, 9, 10); *3*) stimulating mononuclear cells to produce a tissue factor–like procoagulant that converts fibrinogen into fibrin (7); *4*) causing the development of cross mimicry between endothelial and *Hp* antigens; *5*) increasing the aforementioned Hcy, which has been implicated in endothelial damage and neurodegeneration via oxidative injury in these neurodegenerative diseases (7); *6*) producing reactive oxygen metabolites and circulating lipid peroxides also involved in the pathophysiology of AD (7); and *7*) influencing the apoptotic process, which is an important form of cell death in many

neurodegenerative diseases including AD and possibly MCI (7). Notably, *H. pylori* is capable of inducing apoptotic effects through the mitochondrial apoptotic pathway involving activation of the proapoptotic proteins Bax and Bak, activation of certain caspases, or through inducible nitric oxide (7). Nitric oxide is a rapidly diffusing gas and a potent neurotoxin that may contribute to the apoptotic neuronal cell death in degenerative neuropathies (7).

The authors had no conflicts of interest.

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Reply to J Kountouras et al

Dear Sir:

Kountouras et al raise interesting points about the potential relations between *Helicobacter pylori* infection, gastric function, vitamin B-12 absorption, and risk of cognitive impairment. Our research group has not specifically investigated *H. pylori* infection in the Sacramento Area Latino Study on Aging (SALSA), but we did report previously on the cross-sectional association between gastric function and vitamin B-12 status in a nonrandom subsample of study subjects (1). Elevated serum gastrin (≥100 ng/L) is a sensitive predictor of moderate-to-severe atrophy of the gastric body (2). We observed elevated serum gastrin in ≈30% of the SALSA subjects, with a higher percentage (48%) of elevated values in those subjects with a deficient total plasma B-12 concentration (<148 pmol/L). Moreover, we observed a highly significant inverse association between gastrin and plasma vitamin B-12 concentrations ($P < 0.0001$).

The association between *H. pylori* infection and gastric function is complex. Initial, acute *H. pylori* infection results in reduced secretion of gastric acid (3). If the infection persists for several months, gastric acid secretion may normalize or increase (4). If the infection and gastritis are prolonged beyond several months, there is progression to gastric atrophy, and gastric acid secretion is again reduced. This *H. pylori*–induced reduction in gastric acid secretion impairs the capacity to release and absorb vitamin B-12 from animal source foods and may increase the risk of intestinal bacterial overgrowth. The bacteria may compete with the host for dietary vitamin B-12 and reduce the vitamin's bioavailability. Finally, recent evidence has been presented that links previous *H. pylori* infection with subsequent development of autoimmune pernicious anemia (5). The pathogenesis of such an association may involve molecular mimicry by *H. pylori* of gastric mucosal antigen, which allows the organism to "fly below the radar screen" of host immune surveillance with subsequent risk of autoimmune consequences in a susceptible host (6). Thus, it is reasonable to predict that *H. pylori* infection may contribute to low vitamin B-12 status. Data from the National Health and Nutritional Examination Survey show that Mexican Americans 70 y of age and older experience a significantly higher prevalence of infection with *H. pylori* than do white non-Hispanics (74.0% compared with 54.8%) (7). The SALSA study involves a representative sample of the Mexican elderly population in the Sacramento area of California who are demographically similar to national samples of the same age and ethnicity (8). It is therefore likely that the SALSA population carries a high burden of *H. pylori* seroprevalence. It is possible that the relatively high prevalence of low plasma vitamin B-12 (6.5%) we observed in the SALSA cohort may be associated with prevalent infection by *H. pylori*. Whether the influence of gastric atrophy on vitamin B-12 status is of sufficient magnitude to affect cognitive function in the SALSA cohort is open to speculation. Because we did not find an effect of low plasma vitamin B-12 on dementia and CIND outcomes, the link between *H. pylori* and vitamin B-12 may not directly apply to these findings. We also have not yet examined the influence of related medications on vitamin B-12 or on cognitive status.

None of the authors had any conflicts of interest to report.

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The American Journal of Clinical Nutrition

Challenge in the Pathogenesis of Autoimmune Pancreatitis: Potential Role of *Helicobacter pylori* Infection via Molecular Mimicry

Dear Sir:

We read with considerable interest the review by Witt et al[1] on the challenges and advances in the pathogenesis, genetics, diagnosis, and therapy of chronic pancreatitis. Regarding the pathogenesis of autoimmune pancreatitis (AIP), however, the data provided in the article are incomplete and limited to aberrant HLA-DR expression on pancreatic ductal and acinar cells, leading to the presentation of autoantigens to lymphocytes and resulting in an autoimmune response.

In approximately 60%, AIP coexists with other autoimmune diseases such as Sjögren's syndrome (SjS); sclerosing extrahepatic cholangitis (interpreted as a variant of primary sclerosing cholangitis [PSC]); primary biliary cirrhosis (PBC); autoimmune hepatitis (AIH), or other extrapancreatic disorders; recently, gastric peptic ulcer has been reported.[2,3] The histopathologic findings in these extrapancreatic lesions are lymphoplasmacytic inflammation and fibrosis, similar to those in the pancreatic tissue, suggesting a common pathogenesis.[2] The diversity of extrapancreatic lesions with similar histopathologic findings suggests general involvement of the digestive system in this disease, although not fully elucidated; coexistence of pancreatitis with these autoimmune diseases suggests that there may be common target antigens in the pancreas and other exocrine organs, such as the salivary glands, gastrointestinal tract, or biliary tract. Several autoantibodies, such as anticarbonic anhydrase II antibody or antilactoferrin antibody, were frequently detected in patients with AIP. An autoimmune reaction against CA-II or LF via T helper (Th)-1 type CD4+ T lymphocytes might play a role in the development of AIP.[2] The first step in the disease may be an antigenic alteration in pancreatic ductal or acinar cells, such as the aberrant expression of HLA-DR. In turn, CD4+ T cells may recognize the HLA class II complex and autoantigenic peptides such as CA-II, and act as helper or cytotoxic cells probably by inducing apoptosis.[2]

Similarly, *Helicobacter pylori* infection has been strongly associated with peptic ulcer and gastric autoimmunity,[2] and patients infected with *H pylori* have been shown to possess autoantibodies that cross-react with antigens expressed on the gastric mucosa. Moreover, as in the case of AIP, *H pylori* is associated, via molecular mimicry of host structures by its constituents, with the same autoimmune conditions that include PBC, PSC, AIH, and SjS (ie, autoimmune sialadenitis), or hepatitis C virus (HCV)-related liver disease which trigger autoimmune sequelae (AIH and HCV-related SjS).[3] These *H pylori*-related diseases are also characterized by fibrotic changes and/or lymphoplasmacytic inflammations, accompanied by aberrations of T-cell apoptosis that contribute to hepatobiliary or extrahepatic tissue destruction.[2] Interestingly, molecular mimicry of host structures by constituents (such as the saccharide portion of lipopolysaccharides) of *H pylori* is thought to be connected with the development of autoimmune sequelae in autoimmune neuropathies.[4] PBC or possibly AIP, that induce apoptotic damage of neurons, liver tissue, or pancreatic tissue.

Considering these data, we proposed that this organism might trigger AIP through induction of autoimmunity and apoptosis.[2] Prompted by this theory, Guarneri et al[5] sought to identify the potentially cross-reactive human and bacterial protein(s) using amino acid sequence comparison. This technique has been already used in the field of immune-related diseases, yielding significant results; by using in silico protein analysis and search for HLA binding motifs to verify this hypothesis, they found a significant homology between human CA-II and α-CA of *H pylori*, a fundamental enzyme for the bacterium's survival and proliferation in the gastric environment. Moreover, the homologous segments contain the binding motif of the HLA molecule DRB1*0405.[5] Notably, possession of the HLA DRB1*0405-DQB1*0401 genotype confers a risk for the development of AIP.[6] These data strengthen our speculation that gastric *H pylori* infection can trigger AIP in genetically predisposed subjects.

In addition, microcirculatory changes, including vasoconstriction, capillary stasis, decreased oxygen saturation, and progressive ischemia, could lead to local microcirculatory failure, vascular permeability, edema of the gland, and amplification of the pancreatic injury.[6,7] Apart from reactive oxygen metabolites, active granulocytes and macrophages release proinflammatory cytokines (tumor necrosis factor [TNF], interleukin [IL] -1, -6 and -8), arachidonic acid metabolites (prostaglandins, platelet-activator factor, leukotrienes), and proteolytic and lipolytic enzymes that also interact with the pancreatic microcirculation to augment vascular permeability, which induces thrombosis and hemorrhage and leads to pancreatic necrosis. *H pylori* infection could exacerbate these events by promoting platelet and platelet-leukocyte aggregation, releasing large amounts of proinflammatory and vasoactive substances, such as endothelin-1 (a potent constrictor of arterioles and venules), cytokines (IL -1, -6, -8, TNF-α), eicosanoids (leukotrienes, prostaglandins), or stimulating mononuclear cells to induce a tissue factor-like procoagulant activity that converts fibrinogen into fibrin. Whether *H pylori* eradication may indirectly benefit AIP patients by ameliorating the autoimmune sequelae and the apoptotic loss of duct cells and/or acinar pancreatic cells remains to be elucidated.

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Insulin-Like Growth Factor-1 Is Not Essential for the Intestinal Trophic Effects of Glucagon-Like Peptide-2

Dear Sir,

The report by Dube et al[1] proposes that insulin-like growth factor-1 (IGF-1) has an essential role in the intestinal trophic effects of glucagon-like peptide-2 (GLP-2). In studies of a rat fetal intestinal culture system, Dube et al[1] reported a 29% increase in the secretion of IGF-1 after administration of human recombinant GLP-2. Furthermore, their morphometric studies of the intestinal proliferative response to GLP-2 in IGF-1 homozygote (IGF-1$^{+/+}$) and IGF-1 knockout (IGF-1$^{-/-}$) mice suggest that IGF-1 is required for a GLP-2–induced small intestinal proliferative response.

We have studied the effect of long-term (6-week) recombinant GLP-2 administration on circulating IGF-1 and GLP-2 levels, and intestinal mucosal morphology in a piglet model of short bowel syndrome. The results are detailed in Figure 1. We report that treatment with recombinant human GLP-2 results in increased circulating GLP-2 in sham-operated and resected animals, and increased proliferation in the villus of sham-operated and resected animals, and the crypt of resected animals. However, these changes occur despite a significant decrease in circulating IGF-1 levels in both sham-operated and resected, GLP-2–treated animals. Our results indicate that in a piglet model, GLP-2 administration does not result in increased levels of circulating IGF-1, and that IGF-1 is

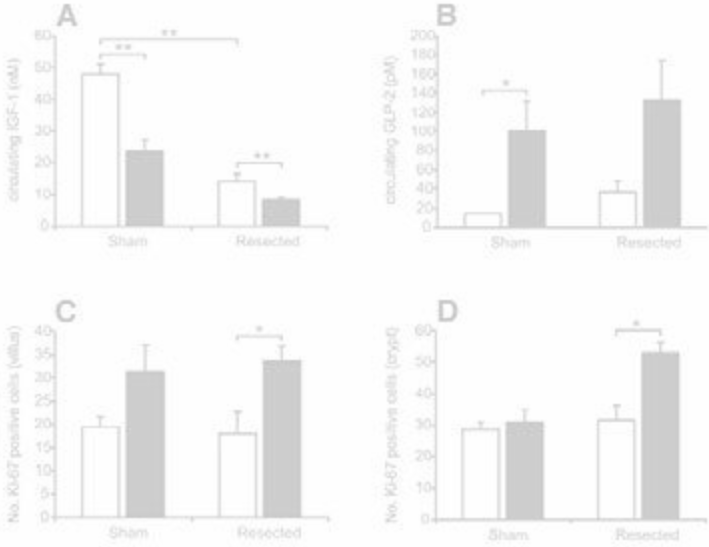

Figure 1. Piglets underwent a sham-operation or 75% small bowel resection and were fed a polymeric infant formula (PIF, □) or PIF supplemented with GLP-2 (800 μg/day, ■) for 6 weeks. (A) Circulating IGF-1 and (B) intact GLP-2 were measured at 6 weeks after surgery, and Ki-67 immunoreactive was used to detect proliferating cells in the villus and crypts of ileal tissue.

Molecular Mechanisms Associated with Aggressiveness of Alpha-Fetoprotein-Positive Gastric Cancer

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To The Editor

We read with considerable interest the paper by Ishigami *et al.* (1) who concluded that "alpha-fetoprotein (AFP)-positive gastric cancer was strongly associated with hematogenous factors such as venous invasion, hepatic metastasis and aggressive biological factors (p53 abnormalities)". However, the authors did not give explanations for the p53 abnormality associated with "high malignant potential such as lymph node and hematogenous metastasis" of this tumor. In particular, the authors did not consider that *Helicobacter pylori* (*H. pylori*) infection may be involved in gastric carcinogenesis and aggressiveness of this tumor through various mechanisms including associations with p53 abnormalities, and indirectly with AFP (2).

The most frequent genetic abnormalities found in gastric cancers tend to be loss of heterozygosity (LOH) of previously described tumor suppressor gene p53 (2). This is a nuclear oncosuppressor protein involved in the maintenance of genomic integrity: DNA damage results in the increased expression of p53, which then causes G1 arrest in the actively cycling cells. It can then induce either factors that facilitate DNA repair, or, if the damage is too great, factors which cause apoptosis (3). Early studies reported that LOH (60-70%) and mutations (38-71%) of the p53 gene are quite frequent in gastric cancer. In addition, p53 mutations are also observed in intestinal metaplasia (38%) and gastric dysplasia (58%), suggesting that mutations of the p53 gene may be an early event and perhaps work together with *H. pylori* infection in the pathogenesis of gastric cancer; *H. pylori* infection increases expression of p53 oncoprotein in the gastric mucosa (4,5). Further evidence for a role of p53 in the early stages of gastric cancer development comes from studies in mice that are hemizygous for p53, which display an increased proliferative response to *H. pylori* infection compared with wild-type mice. Increased proliferation is correlated with an increased risk of developing gastric malignancy (6).

From another viewpoint, recent studies indicate that the inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF) and the tumor suppressor p53 are fundamental play-markers of the angiogenic process. Overexpression of iNOS and VEGF has been shown to induce angiogenesis in tumors, whereas p53 suppresses angiogenesis by down-regulating VEGF and iNOS. On the other hand, mutations of the p53 gene have been thought to upregulate VEGF and possibly iNOS; p53 protein accumulation and increased expression of iNOS and VEGF might be responsible for gastric carcinogenesis and aggressiveness of gastric tumor (2). In this regard, higher expression of VEGF isoform C (VEGF-C) might be one explanation for the poorer prognosis of AFP-producing gastric cancers (7). Moreover, *H. pylori* upregulates VEGF expression in gastric epithelial cells (8), and iNOS contributes to *H. pylori*-associated gastric carcinogenesis in mice (9). From a practical point of view, it has been demonstrated that preoperative intra-arterial chemotherapy could enhance the apoptosis of gastric cancer cells, decrease the level of p53 expression and keep the patients for a longer survival. Taken together, these data suggest that inactivation of p53 is essential in the early pathogenesis of gastric cancer, and, moreover, it might be related with the tumor aggressiveness (2).

Importantly, *H. pylori* may also activate the c-Met, thereby promoting gastric cancer (10). The c-Met gene, a proto-oncogene member of the tyrosine kinase growth factor receptors, is amplified in 10.2% and overexpressed in 46.1% of gastric cancers. Its ligand, hepatocyte growth factor (HGF)/scatter factor, is also overexpressed in 67% of gastric cancers (11). Amplification of the c-Met gene is associated with increased depth of tumor invasion, lymph node and liver metastases and decreased survival (11). Moreover, a higher frequency of c-Met expression is observed in AFP-producing gastric cancer and is associated with decreased apoptosis, high incidence of liver metastasis and poor prognosis. A higher expression of c-Met might be one further explanation for the poorer prognosis of AFP-

Hepato-Gastroenterology 2007, 54:329-330

producing gastric cancers, because HGF and its receptor, c-Met, are known to induce mitosis and cell movement and to promote tumor progression (12,13).

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Otolaryngology–Head and Neck Surgery (2007) 136, 334-335

LETTER TO THE EDITOR

The role of gastric *Helicobacter pylori* infection in laryngopharyngeal reflux disease

The article by Ercan et al[1] presents a first appraisal of the potential relationship between *Helicobacter pylori* (*H. pylori*) infection and laryngopharyngeal reflux, attempting to compare their findings with the available controversial published data on gastroesophageal reflux disease (GERD), with respect to the potentially protective pathogenetic role of *H. pylori* in GERD. We believe that the increasing incidence of GERD complications after *H. pylori* eradication may be explained not just by the diminishing prevalence of *H. pylori* infection, but rather by healing of *H. pylori*-associated peptic ulcer disease, which coexists with GERD.[2] Up to 60% of the patients with duodenal ulcer present with concurrent GERD symptoms. Thus, eliminating peptic ulcer disease unmasks previously occult GERD.[2]

This statement is further supported by retrospective studies in large cohorts (approx. 21,000 patients), showing that the decrease in *H. pylori* infection during the period 1996-2002 coincides with a decrease in the prevalence of peptic ulcer disease and an increase in GERD, while reappearance of GERD after *H. pylori* infection seems to be rare.[3] These data are also supported by more recent relative studies.[4] Moreover, the perception that the "protective" role of *H. pylori* in GERD is mediated via induction of the sequence: *H. pylori*-associated atrophic gastritis–hypochlorhydria–decrease in acidic reflux–not development of GERD seems to be unconvincing because *H. pylori*, via the same sequence leading to hypo- or achlorhydria, would be expected to protect against duodenal ulcer disease, which is clearly irrational given the tenet: no acid, no ulcer.[5] Recent great body of evidence is in accordance with the last concept, showing that *H. pylori* is not and never was "protective" against anything, including GERD.[6]

On the contrary, the following rationale seems to exist: the appearance of GERD depends on the esophageal acid exposure, and its symptomatology is related to acid hypersecretion, condition that predisposes to peptic ulcer disease. Given that the vast majority of peptic ulcer cases are caused by *H. pylori* infection, the bacterium could therefore dynamically also promote GERD development by inducing esophageal acidity.[5]

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Reply to: The role of gastric *Helicobacter pylori* infection in laryngopharyngeal reflux disease

We thank Kountouras et al for their comments on our paper. In the letter the possible pathophysiology of the relationship between *Helicobacter pylori* (*H. pylori*), acid secretion, and gastroesophageal reflux disease (GERD) was remarked upon commenting from literature. Even though there are controversies involving the relationship between *H. pylori* and GERD, it is a well-known fact that the role of *H. pylori* in GERD may be due not only to its presence but to the type of gastritis caused: antrum-predominant or pangastritis.[1] This is due to the location of the gastritis associated with bacterium where presence in the antrum or corpus determines the amount of acid secretion which affects symptoms related to GERD. The above mentioned data were thoroughly discussed in our article.

Predominat corpus gastritis associated with *H. pylori* leads to a decrease in acid secretion, thus masking symptoms related to GERD. This mechanism is perceived by some authors to be "protective."[2] In an article by Graham,[3]

he states that the term "protective" leads to a misleading perception and may be scientifically incorrect. The debate on this concept is not relevant regarding our article. Even though we mentioned the "protective" role of *H. pylori* in GERD in reference to the masking of symptoms, we did not construct our article regarding this statement. The results of our study showed that there are no statistically significant differences between laryngopharyngeal reflux (LPR) and gastric *H. pylori*.

On the other hand, the combination of *H. pylori* eradication to empiric[4] treatment initiated for LPR patients is a new issue and has not yet been researched. In countries such as our own where seropositivity for *H. pylori* is around 60%, the rationale of eradication of *H. pylori* in the treatment of LPR is a question that should be answered. In our article we hoped to light a path for the further understanding of this topic.

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Reply to: Coblation lingual tonsillectomy

We appreciate the favorable comments of Dr Mair[1] regarding using Coblation™ (Arthrocare Corp, Sunnyvale, CA) for removal of lingual tonsils. The tool assists in easing a previously difficult procedure by excising tissue in a relatively bloodless field, reducing trauma, and allowing improved visualization and safe tissue removal in a hard to access field.

Dr Mair correctly describes the sometimes difficult and tedious nature of surgical exposure in this region and we are excited about his described method of exposure. However, his negative comments about the cumbersome nature of direct laryngoscopy are somewhat overstated. For many cases and surgeons, the use of the laryngoscope will continue to be appropriate. Examples may include those with small but significant lingual tonsils, where lingual tonsillectomy is performed combined with other pharyngeal procedures. A hand held laryngoscope provides excellent exposure for rapid removal of tissue from the valleculae and base of tongue without the need or expense of endoscopes. In others with a small oral cavity and relative macroglossia, tongue traction and protrusion may not adequately expose the hypopharynx. In these patients, an instrument such as a laryngoscope is needed to displace the tongue out of the field. Lastly, the use of the suspension laryngoscope allows for bimanual surgery which may be needed for selected patients. Ultimately, it is not the tool but the exposure and visualization that is critical for successful lingual tonsillectomy. We thank Dr Mair for his important contribution.

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Eradication of *Helicobacter pylori* might halt the progress to oesophageal adenocarcinoma in patients with gastro-oesophageal reflux disease and Barrett's oesophagus

Previously rare, oesophageal adenocarcinoma (OA) is now the most common oesophageal malignancy in Western countries, including the United States and England, its incidence increasing faster than any other cancer [1]. Barrett's oesophagus (BO) is a complication of long-standing gastro-oesophageal reflux disease (GORD) and well-recognised premalignant condition playing a pivotal role in OA development [1]; GORD plays a crucial role in the pathophysiology and the clinical identification of BO which represents the most serious histologic consequence of chronic GORD [1]. In this regard, our recent data show that *Helicobacter pylori* (*H. pylori*) is frequent in GORD and even in non-endoscopical reflux disease (NERD) [2,3], and *H. pylori* eradication leads to better control of GORD symptoms and improves oesophagitis [2]. Other authors [2] also reported improvement in reflux symptoms following *H. pylori* treatment. A great body of recent evidence further potentiates the concern that *H. pylori* is not "protective" against GORD [2].

H. pylori may contribute to GORD pathogenesis by several mechanisms including release of several mediators, cytokines and nitric oxide which may adversely affect the lower oesophageal sphincter (LOS); direct damage of the oesophageal mucosa by bacterial products; increased production of prostaglandins that sensitise afferent nerves and reduce LOS pressure; and augmented acidity (by gastrin release) that exacerbate GORD [2].

Gastrin is an oncogenic growth factor contributing to oesophageal, gastric and colon carcinogenesis; gastrin stimulates receptor-mediated proliferation of human OA cells, shows antiapoptotic activity through upregulation of Bcl-2 and survivin and upregulates cyclooxygenase (COX)-2 expression. *H. pylori* infection activates NF-κB, an oxidant-sensitive transcription regulator of inducible expression of inflammatory genes such as COX-2, which regulates gastrointestinal cancer cell growth and proliferation. In particular, *H. pylori* infection induced NF-κB and COX-2 expression in oesophageal epithelial cells, playing a role in inflammation and tumorigenesis in the oesophagus [4]. Moreover, recent evidence indicates that: (a) *H. pylori* infection prevalence is high in BO; (b) cagA-positive *H. pylori* infection is not associated with decreased risk of BO; (c) the expected incidence of OA with persistent *H. pylori* infection is higher than that of OA after eradication of infection [5]; and (d) *H. pylori* induces Ki-67 expression and increased oesophageal expression of Ki-67 observed in BO patients compared with GORD controls. Ki-67 proliferation fraction increases significantly from

normal squamous epithelium to BO – dysplasia – OA.

Therefore, we speculate that *H. pylori* might be involved in the GORD-BO-OA sequence and its eradication might inhibit the progress of this sequence.

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Leflunomide as an antiatherogenic drug

Dear Editor,

Although several drugs so far have been successful in the prevention of atherosclerotic vascular disease, it has been recently pointed out that the burden of cardiovascular morbidity and mortality is not likely to change in the near future unless fundamentally new therapeutic tools are found [1].

At this regard we speculate that leflunomide (LEF), a disease-modifying antirheumatic drug which is widely used in patients with rheumatoid arthritis (RA) [2], could also ameliorate vascular function by affecting several crucial mechanisms involved in the vessel wall pathobiology. Firstly, it has been shown that LEF has the ability to inhibit the nuclear factor kappaB signal transduction pathway [3], which is deemed to be critical in the development of a proinflammatory and proatherosclerotic phenotype in endothelial cells [4]. In addition, it has been demonstrated that leflunomide may reduce the subendothelial migration of peripheral blood mononuclear cells – an important event in early atherogenesis – by decreasing the cell surface expression of intercellular adhesion molecules [5]. Third, LEF may cause functional impairment of antigen-presenting dendritic cells [6], whose activation has been recently demonstrated at sites of vascular pathology [7]. Altogether, these data may lead to the hypothesis that LEF could exert important vasculoprotective effects. In keeping with our hypothesis, preliminary results obtained in RA patients indicated that LEF use was associated with a significantly reduced risk of developing myocardial infarction [8].

Since the molecular actions of LEF indicate that its vasculoprotective effects may be independent of its antirheumatic properties, clinical testing of this compound as a cardiovascular medication in the general population may be worthwhile. Does treatment with LEF reduce the risk of ischemic cardiac events in the general population, and is it similar to that observed in RA? Does treatment with LEF have any beneficial effect on markers of endothelial function – such as flow-mediated vasodilation – in people with cardiovascular diseases? Is the tolerability profile of LEF in the general population similar to that observed in RA? We believe that clinical trials would be useful to answer to these

Normal-tension glaucoma and Alzheimer's disease: *Helicobacter pylori* as a possible common underlying risk factor

Dear Editor,

In his correspondence Dr. Wostyn hypothesized that excessive Valsalva may be a common risk factor underlying normal-tension glaucoma (NTG) and Alzheimer's disease (AD) [1]. Another common risk factor involved in the pathogenesis of AD and glaucoma appears to be *Helicobacter pylori* (*Hp*) infection [2–7].

Accumulating evidence indicates a presence of pathogenetic associations between NTG and endothelin-dependent vascular dysregulation [2,3]. In addition, impaired ocular blood flow due to blood hyperviscosity (increased platelet aggregation on atheromatous plaques) and cytokines are also involved in NTG pathogenesis [2,3]. Moreover, autoimmune mechanisms (causative role of autoantibodies) may be responsible for peripheral neuropathies and glaucomatous damage [2].

In this respect, *Hp* has been implicated in a variety of extradigestive vascular conditions including functional vascular disorders, hypertension, ischaemic heart disease, and ischaemic cerebrovascular disorders, also detected in glaucoma and AD and contribute to their clinical manifestations [3,4]. More specifically, in the nervous system, *Hp* is thought to be associated with the development of autoimmune sequelae observed in peripheral neuropathies, and glaucomatous optic neuropathy; recent evidence also suggests the possible presence of anti-neuronal antibodies and autoimmunity-induced cell death in AD [4,5].

Taking into consideration the aforementioned data, a theoretical relationship between *Hp* infection, glaucoma and AD seems to exist. Recently, a higher prevalence of *Hp* in glaucoma and AD patients has been reported, suggesting a potential association between *Hp* infection and these diseases [4,6]. Furthermore, in a subsequent study we documented a beneficial effect of *Hp* eradication upon glaucoma progression [3,6]. A beneficial effect of *Hp* eradication upon AD clinical progression was also observed (Kountouras et al. unpublished data). Moreover, anti-*Hp* IgG antibody levels were significantly increased in the aqueous humour and serum of patients with glaucoma reflecting the severity of glaucomatous damage [6].

Hp may influence the pathophysiology of glaucoma and AD by promoting platelet and platelet–leucocyte aggregation [6], also involved in the pathophysiology of glaucoma and AD [2–4,6]; releasing large amounts of proinflammatory and vasoactive substances, eicosanoids, and acute phase proteins [6], involved in glaucoma and probably AD [2–4,6]; stimulating mononuclear cells to produce a tissue factor-like procoagulant that converts fibrinogen into fibrin [6]; causing the development of cross mimicry between endothelial and *Hp* antigens [6]; producing reactive oxygen metabolites and circulating lipid peroxides [6], involved in the pathophysiology of glaucoma and AD [2–4,6]; influencing the apoptotic process in glaucomatous neuropathy and AD [2, 4–6].

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A wake-up call: Are degenerative diseases provoked by some of our normal food constituents? A case for nutrigenomics

Today most patients diagnosed with a degenerative disease such as Alzheimer's are over 50 years old – so when we receive the certain diagnosis of a degenerative disease, the effects of the disease have probably been in our body at the molecular level for several years, possibly for decades.

Can there be normal food constituents that, taken over a life long period, enhance the possibility of getting a degenerative disease?

There has been and is being performed a large amount of research concerning the major degenerative diseases, such as Alzheimer's, but still the causes of the diseases are not clear. We have, though, a clear picture of the symptoms of the diseases in humans at the final stage obtained by autopsy.

In the following Alzheimer's disease is taken as an example. Although there are genes that are connected to the development of Alzheimer's, unfortunately only around 5% of patients diagnosed with Alzheimer's have a genetic explanation, the remaining 95% have what is currently denoted sporadic Alzheimer's.

Some of the symptoms of Alzheimer's seem to be clearly associated with glucose.

1. Senile plaques, the central core containing amyloid β-peptide (Aβ). Aβ inhibits glucose transport in neurons [1].
2. Reduced concentration of GLUT 1 (facilitative glucose transporter) and GLUT 3 [2,3].
3. Increase in the number of RAGE-expressing microglial cells. RAGE: The receptor for advanced glycation end products (AGE) [4,5].

After having entered the brain through GLUT 1 situated in the blood brain barrier, glucose can enter the neurons through GLUT 3, 4 and 8 and the astrocytes through GLUT 1 and 2 [2].

The astrocyte will upon pathological activation provide immune functions in the brain, but an astrocyte that has been activated cannot perform all the protective and nourishing functions for the neuron as it can when not activated. This means that if destruction of infected tissue and repair are not correctly coordinated, inflammation can lead to persistent tissue damage.

Coordination would probably involve the regulation of the amount of glucose entering through the GLUTs. One of the ways to regulate the amount of glucose is to regulate the density of the GLUTs. All aspects of this are not clear today.

From the above there seems to be a possible biochemical connection between a degenerative disease and a common food constituent.

To prove this experimentally a combination of in vitro immunological measuring methods and in vivo imaging techniques has to be applied. All of these methods will need fine tuning to this specific task. Methods within nutrigenomics combined with bioinformatics should also be applied.

Quite possibly elucidation of the biochemical connection will aid in finding unknown genetic couplings to a specific degenerative disease.

A consequence of confirming this hypothesis is that degenerative diseases hopefully can be prevented/delayed by consuming a more appropriate combination of food constituents. The appropriate combination will vary for each individual, being dependent not only upon the genetic composition of the individual but also upon the combination of

CORRESPONDENCE

Apolipoprotein E Polymorphisms in Patients with Primary Open-Angle Glaucoma

EDITOR:

WE READ WITH GREAT INTEREST THE ARTICLE BY ZETTERberg and associates, who considered a possible common pathogenetic mechanism for primary open-angle glaucoma (POAG) and Alzheimer disease (AD) without involvement of apolipoprotein E.[1] A common underlying risk factor involved in the pathogenesis of AD and POAG seems to be *Helicobacter pylori* infection.[2–6]

Current *H. pylori* infection induces irregular 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 the apoptotic neural tissue damage observed in POAG and other neurodegenerative diseases, including AD.[2–6]

Although the initial triggering mechanism(s) that induce aqueous outflow obstruction or increase the susceptibility of ocular tissues to glaucomatous damage remain unknown, accumulating evidence suggests pathogenetic associations between glaucoma, endothelin-dependent vascular dysregulation, and impaired ocular blood flow resulting from blood hyperviscosity.[2,3] Cytokines may also be involved in the pathogenesis of POAG.[2,3] Moreover, autoimmune injury to the optic nerve may occur directly by autoantibodies or indirectly by way of a mimicked autoimmune response to a sensitizing antigen, which in turn damages retinal ganglion cells. Specific antibodies are found in increased levels in the sera of glaucoma patients; these antibodies are capable of killing retinal cells experimentally.[6]

Correspondingly, in the nervous system, *H. pylori* is thought to be associated with the development of autoimmune sequelae observed in peripheral neuropathies, Guillain-Barré syndrome, and glaucomatous optic neuropathy, where autoantibodies to specific neural targets have been found to impair native neural function by inducing nerve tissue damage possibly by apoptosis.[6] With respect to the central nervous system exclusively, recent evidence also suggests the possible presence of antineuronal antibodies and autoimmunity-induced cell death in AD.[5]

In this regard, we reported a higher prevalence of *H. pylori* infection in Greek patients with POAG, defined as ocular AD, and AD.[2–6] In addition, the titer of anti-*H. pylori* antibody in aqueous humor may reflect the severity of glaucomatous damage.[6] Moreover, *H. pylori* eradication may influence POAG parameters positively, suggesting a possible causal link between *H. pylori* and POAG[4] and possibly AD.[5]

H. pylori infection may influence the pathophysiologic features of POAG and AD by one of the following mechanisms:

1. Promoting platelet and platelet–leukocyte aggregation. Platelet activation and aggregation also have been proposed to play pathophysiologic roles in the development of glaucoma and AD.[2–5]
2. Releasing large amounts of proinflammatory and vasoactive substances, such as cytokines (interleukin [IL]-1, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor α, interferon-γ), eicosanoids (leukotrienes, prostaglandins catalyzed by cyclooxygenase enzymes), and acute phase proteins (fibrinogen, C-reactive protein) involved in a number of vascular disorders, including glaucoma and probably AD.[2–5]
3. Stimulating mononuclear cells to produce a tissue factor-like procoagulant that converts fibrinogen into fibrin.[2–5]
4. Causing the development of cross-mimicry between endothelial and *H. pylori* antigens.[2–6]
5. Producing reactive oxygen metabolites and circulating lipid peroxides that also have been involved in the pathophysiologic characteristics of glaucoma and AD.[2–5]
6. Influencing the apoptotic process that may also be an important form of cell death in many relative neurodegenerative diseases, including glaucomatous neuropathy and AD.[2–6]

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REPLY

WE THANK DR KOUNTOURAS AND ASSOCIATES FOR THEIR interest in our article and for their comments. We strongly agree that primary open-angle glaucoma (POAG) and Alzheimer disease may share underlying pathogenic mechanisms and that research in this field may be beneficial for developing new therapeutic strategies. Their hypothesis about *Helicobacter pylori* being a common underlying risk factor for both POAG and Alzheimer disease is interesting, although conflicting data have been presented.[1,2] However, vascular and immunologic mechanisms, both of which can be triggered by an *H. pylori* infection, have been implied in neurodegenerative conditions in general and in Alzheimer disease and POAG in particular.[3,4]

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Relationship of Hemoglobin A1c with the Presence and Severity of Retinopathy upon Initial Screening of Type II Diabetes Mellitus

EDITOR:

WE WOULD LIKE TO EXPRESS OUR CONCERNS REGARDING the article by Maa and associates on the relationship between glycosylated hemoglobin (HbA1c) and diabetic retinopathy.[1] The authors conclude that "HbA1c levels appear to have no meaningful predictive value for the presence or severity of diabetic retinopathy," based on their inability to find an association between HbA1c and retinopathy from a small retrospective survey. Although we understand the intent of their article was, in part, to inform eye care providers that glycosylated hemoglobin levels are not a good substitute for examinations to detect vision-threatening retinopathy, we believe that their conclusion inadvertently may mislead readers into believing that glycemic control is not related to diabetic retinopathy.

The most consistent and important risk factor for diabetic retinopathy is glycemic control. This is well documented in epidemiologic studies such as the Wisconsin Epidemiological Study of Diabetic Retinopathy, in which participants with HbA1c levels of more than 12% had a three-fold higher risk of retinopathy than those with levels of less than 12%. This finding was independent of diabetes duration and other risk factors.[2] Higher HbA1c level also was shown independently to predict both the incidence and progression of diabetic retinopathy in other studies.[3,4] Two landmark clinical trials conclusively confirmed the importance of glycemic control in reducing the incidence and progression of diabetic retinopathy. The Diabetes Control and Complications Trial in type 1 diabetes showed that after 6.5 years of follow-up, patients with tight glycemic control had a 75% lower incidence of retinopathy and a 50% lower rate of progression to more severe retinopathy as compared with patients randomized to conventional treatment.[5] The United Kingdom Prospective Diabetes Study in type 2 diabetes reported that after 12 years of follow-up, tight glycemic control was associated with a 21% reduction in retinopathy progression and a 29% reduction in the need for laser treatment.[6] These studies have provided high-quality evidence validating the usefulness of HbA1c as a predictor of diabetic retinopathy development and progression.

The failure to find a significant association between glycemic control and diabetic retinopathy in the study by Maa and associates may be the result of a number of different limitations. First, insufficient power from the small sample size might have limited the ability to detect an association. Second, the study sample was highly selective: more than half of the patients examined were excluded from the study. Third, the ascertainment method for retinopathy is unclear.

Although we recognize the authors' primary intention was to convey a clinically valid message that HbA1c measurement cannot (and should not) replace routine ophthalmoscopic examination to screen for retinopathy and assess its progression, we believe that their final message may mislead readers.

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Parikh's formula to minimize errors in calculating expected date of delivery

Expected Date of Delivery (EDD) in pregnant females with typical 28-day menstrual cycle is calculated by counting back three calendar months from the first day of the last menstrual period (LMP) and then adding one week [1]. In general population though the duration of menstrual cycle averages 28 days but it ranges from 20 to 45 days even in normal women [2]. EDD calculated by Naegele's rule will have an error depending on the duration of cycles. There is need for a formula which takes into consideration this fact and gives EDD incorporating duration of cycles as a variable.

In any cycle the period from ovulation to menstruation i.e. leuteal phase is more or less constant at 14 days [3]. In longer or shorter cycles it is the duration of follicular phase, which varies and will be equal to duration of previous cycles minus 14 days. For calculating EDD from Last Menstrual Period (LMP), duration of follicular phase (Duration of previous cycles − 14 days) should be added to average length of human gestation i.e. 266 days (38 weeks or 9 Calendar Months −7 days) from the day of ovulation [1,4]. Author proposes Parikh,s formula i.e. EDD = LMP + 9 Months + (Duration of Previous Cycles − 21 days) as an alternative to Naegele's rule.

If Naegele's rule is used in patients with longer cycles the EDDs are figured incorrectly. If facilities for ultrasonographic estimation of gestational age are not available (as in most of the rural areas in developing world), the patient may be pressured into various unnecessary interventions at term when she is not really at term. Patient usually lands up into induction of labor which when done on an unripe cervix has a very high failure rate, can be quite painful, and often needs a caesarian section because of failure to progress. Such babies born by induction due to incorrect dating may be born earlier than nature intended, and can have immature lungs and other problems, needing special care. If the woman's EDD is set incorrectly, her AFP (alpha-fetoprotein) test for birth defects may come back with a false positive. She may be pushed into unnecessary fetal testing at term and may be pressured into inducing labor too early in the pregnancy, when she is physically and emotionally very vulnerable to suggestions. These shortcomings can be overcome by supplanting Naegele's rule with Parikh's Formula which is more correct scientifically.

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Mitogen-activated protein kinase (MAPK) intracellular signalling in the aqueous humour activated by *Helicobacter pylori* may have a role in glaucoma

Dear Editor,

We read with interest the article by Belt-Yannai and Shmulevich [1], who proposed that mitogen-activated protein kinases (MAPKs) present in the aqueous humour are involved in glaucoma pathology. Specifically, the authors suggested that MAPKs proteins mediate the release of peptides by the trabecular meshwork that affect the drainage system thereby increasing the intraocular pressure

(IOP). However, with regard to defining glaucoma, the earlier emphasis on numerical IOP values is no longer in favour because some patients with glaucoma may initially present with elevated IOP, whereas others may demonstrate glaucomatous damage by optic nerve examination or visual field testing with an IOP less than the traditional cut-off of 21 mmHg (i.e., normal-tension glaucoma). Therefore, the authors' intention to test their hypothesis by injection of several MAPK cascade inhibitors and evaluation of the effect on the IOP *in vivo* may not lead to accurate results.

Recently, we provided evidence for the role of *Helicobacter pylori* infection (*Hp*-I) in the pathophysiology of glaucoma having found increased levels of *Hp*-specific IgG antibodies in the aqueous humour, the titre of which might reflect the severity of glaucomatous damage, in patients with primary open-angle and exfoliation glaucoma [2]. We further proposed the existence of an apoptotic link in the pathophysiology of both diseases, which may involve *Hp*-induced variable apoptotic signals including generation of reactive oxygen species (ROS) and circulating lipid peroxides [3]. In this context, Beit-Yannai and Shmulevich provide an interesting hypothesis that MAPKs present in the aqueous humour may be a novel signal involved in glaucoma pathology, which is in accordance with our evidence on the role of *Hp*-I in glaucoma. Interestingly, it has been shown that this bacterium induces activation of the MAPKs extracellular signal-regulated protein kinase (ERK) 1/2, and MAPK/ERK kinase (MEK) 1/2 [4], mediated at the level of the interleukin (IL)-8 promoter; *Hp* induces IL-8 production leading to neutrophil migration and activation, ROS generation and oxidative tissue damage [4,5]. Furthermore, as Beit-Yannai and Shmulevich also declare, the components of the ERK–MAPK pathway in the trabecular meshwork *in vitro* are dramatically affected by exposure to tumour necrosis factor-α, a pro-inflammatory cytokine also released by *Hp* [3]. Therefore, it would be interesting if Beit-Yannai and Shmulevich could assess the potential delay in the progress of the glaucomatous damage after administration of known inhibitors of *Hp*-induced activation of ERK, such as genistein [6].

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Targeting E-cadherin—catenin complex and eradicating *Helicobacter pylori* may be effective in managing inflammatory bowel disease and its complications

Down-regulation of E-cadherin contributes to enhanced neutrophil transmigration and neutrophil-mediated damage in active inflammatory bowel disease (IBD). Delayed neutrophil apoptosis accompanied by reduction of pro-caspase-3 expression in IBD neutrophils is a feature of persistent inflammation and neutrophil oxidative machinery-mediated damage associated with IBD development [1,2]. Neutrophils from IBD patients have demonstrated reduced spontaneous apoptosis compared to cancer patients. Mesenteric venous serum of IBD patients contributed to this delay, which contained higher levels of neutrophil chemokine interleukin (IL)-8 [2]. Therefore, alterations in cell death mechanisms may lead to the inflammatory process persistence in IBD [2].

E-cadherin abnormal expression also correlates with intraepithelial T-lymphocytes (TLs) involved in IBD pathogenesis. Moreover, IL-12 that causes abnormal E-cadherin regulation can mediate TL-resistance against apoptosis, resulting in prolonged cytokine production by Fas-expressing Th1 effector cells and, ultimately, damaging the gut by activation of matrix metalloproteinases [2]. TL-resistance against apoptosis can extend their lifespan, leading to perpetuation of chronic inflammation with potential tumourigenic effect in the intestine (IBD and adenocarcinoma) [2]. In this regard, high levels of proinflammatory leukotrienes (LTs) and up-regulated expression of cyclooxygenase (COX)-2, characteristic of inflammation, have been implicated in cell survival and early colon carcinogenesis; LTs cause a time- and dose-dependent increased expression and/or membrane accumulation of β-catenin, COX-2 and Bcl-2, as well as prostaglandin E_2 production, and the effects of LTs on these transformation-associated proteins correlate with the ability of LTs to reduce programmed cell death [2].

Helicobacter pylori (Hp)-specific TLs are increased in the intestine of active IBD patients. By using a TL cloning technique, it was shown that the majority of Th1-TLs were specific for *Hp* antigens. A Th1 predominance associated with high IL-12 expression was also found at both clonal and immunohistochemical level in the intestine of IBD patients, further supporting the contribution of inappropriate TL-mediated perpetuation of the IBD chronic inflammatory process ensuing tumourigenesis. In this regard, gastrin, induced by *Hp*, can act as promoter of cell proliferation and differentiation (mainly by inducing COX-2 overexpression) in different gastrointestinal tract sites including colon. Gastrin can induce PI3-kinase-mediated tyrosine phosphorylation of E-cadherin and β-catenin, and delocalisation of β-catenin into the cytoplasm, thereby contributing to neoplasia [3]. Apart from gastrin induction, *Hp* infection mostly frequent in colonic tumour tissues, is accompanied by increased cell proliferation and impaired apoptotic processes [4], and, possibly, by bone-marrow–derived stem cells (CD34+) recruitment that ultimately facilitate colon cancer progression [5]. We therefore propose that *Hp* eradication might inhibit IBD-related or not colon neoplasia [1].

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Analysis of mitochondrial DNA by PCR/DHPLC as a diagnostic tool to differentiate schistosomes species and strains

Dear Editor,

Blood flukes in the genus *Schistosoma* are important human parasites in tropical regions that may cause mortality and severe morbidity in endemic populations. The adult schistosomes infecting humans may live either in the mesenteric venules (*Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma mekongi*, *Schistosoma intercalatum*) or in the venules of the genitourinary tract (*Schistosoma haematobium*) [1].

Given the lethality of invasive *Schistosoma* infections, rapid and reliable identification of parasites at the species and strains level is crucial for correct treatment of patients with schistosomiasis [2]. However, currently available laboratory methods for the diagnosis of this parasitosis often lack enough sensitivity and specificity [3].

We propose that such limitations may be overcome by the use of denaturing high–performance liquid chromatography (DHPLC)–based techniques to allow rapid and high–resolution analysis of PCR products from *Schistosoma* spp. In such a way, the recent sequencing of the mitochondrial genome of *Schistosoma* spp. [4] may provide a unique opportunity for the design of PCR primers for the specific and sensitive detection of schistosomes in human samples. Accordingly, mitochondrial DNA sequences – characterized by a high interspecies and interstrain variability [4] – may be used to generate PCR products for *Schistosoma* spp. ensuring very high specificity.

Such PCR fragments may be analyzed with the use of the WAVE microbial analysis system [5], resulting in peak profiles displaying specific signals for each strain. Each peak could be evaluated using reference strains from *S. mansoni*, *S. japonicum*, *S. mekongi*, *S. intercalatum*, and *S. haematobium*.

We believe that such a PCR/DHPLC approach can be suited to identify *Schistosoma* species from blood, urine or faecal samples in patients with suspected schistosomiasis. Moreover, analysis of schistostomal mitochondrial DNA by means of our PCR/DHPLC assay may provide a unique tool to shed more light on the biological complexity of human schistosomes. This could also be of aid to identify novel molecular interventions and diagnostic targets against such parasites.

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Letters

A critique on the possible protective role of Helicobacter pylori infection in childhood asthma

Sir,

Newer data have emerged attempting to address the possible inverse relationship between Helicobacter pylori (H. pylori) infection and the risk of asthma in children or young adults[1].

Two pathophysiologic mechanisms have been speculated:

1) Because a) a percentage of asthma might be associated with gastro-esophageal reflux disease (GERD); and b) according to a few initial studies, H. pylori eradication might also deteriorate GERD, it is deduced that H. pylori may protect against asthma by protecting against GERD.

2) The potential protective role of H. pylori is consistent with the "hygiene hypothesis" that microbial infections during early childhood may prevent or diminish asthma.

Rebutting the first argument, we believe that it is confusing because if H. pylori "protects" against GERD by inducing corpus gastritis associated with reduced acidity, then corpus gastritis also protects against duodenal ulcer disease. Therefore, using the same argument one could state that H. pylori protects against duodenal ulcer disease, statement that is irrational[2]. The studies that investigated the association between H. pylori and asthma relied on serology for detection of H. pylori infection and have not evaluated corpus gastritis or atrophy. Thus, this pathophysiologic mechanism remains clearly a speculation.

Although the second pathophysiological mechanism proposed might be reasonable and merits future elucidation, not recommending eradication of H. pylori even in symptomatic children or young adults is not justified. However, in older ages, H. pylori has been associated with various upper gastrointestinal diseases and extradigestive conditions including functional vascular disorders caused by vascular dysregulation (e.g., Raynaud phenomenon and migraine), ischemic heart disease, ischemic cerebrovascular disorders, glaucoma, Alzheimer disease, mild cognitive impairment, multiple sclerosis, and some autoimmune conditions such as Sjögren syndrome[3-5], possibly necessitating its eradication.

We have recently acknowledged a number of specific factors, such as increased contact with other children, pets or farm animals, and cross-infections between siblings in early life that may decrease the severity or protect against the progression of asthma in children. In rural areas, children are often exposed to these natural allergens and, according to our data collected from children living in an agricultural area[6,7], this might be the reason why asthma did not dramatically deteriorate in any of our patients, and was managed entirely in the primary care center with very few hospital referrals or admissions.

Summarizing, the role of H. pylori infection in protecting against childhood asthma remains unclear, although persistence of this infection is likely to cause more problems later in life, rather than potentially protect against asthma and its complications.

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