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Oral Medicine

Outcome following treatment for *Helicobacter pylori* in patients with recurrent aphthous stomatitis

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OBJECTIVE: The aim of the current study was to investigate any association of *Helicobacter Pylori* (HP) in recurrent aphthous stomatitis (RAS) and the effect of eradication of the microorganism in the clinical course of the disease.

STUDY DESIGN: Forty-eight patients with RAS were included in the study. Twenty-six were women and 22 men, of average age 41.3 ± 2.44. Thirty-four out of these 48 patients were HP positive and the rest 14 who were negative were used as a control group. The diagnosis of HP infection was based on the detection of specific immunoglobulin G (IgG), and immunoglubulin A (IgA) antibodies using the enzyme-linked immunoabsorbent assay technique in the serum and the saliva of the patients. In all HP carriers an eradication therapy was administered. After a 2-month period the patients were checked for HP status, using ^{13} C-UBT. The follow up period was 6–12 months following the eradication therapy.

RESULTS: At entry patients with HP infection suffered from more severe symptoms compared with HP negative patients ($P < 0.05$). After the administration of HP eradication therapy, patients who had become negative showed a remarkable improvement (62.5%) with reference to recurrence of RAS as well as to symptom intensity. In 29.2% of patients symptoms had disappeared and in 33.3% of patients there was a decrease in both the frequency of recurrence and the intensity of symptoms. After the eradication treatment, the periods between recurrence of RAS in patients who had become negative were statistically significantly longer compared with those before treatment ($P < 0.001$). Another important observation was that patients who became negative after eradication therapy were of comparable clinical status with those who were HP negative from the beginning of the study ($P > 0.05$).

CONCLUSIONS: These findings support the concept of a potential association between RAS and HP.
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Keywords: *Helicobacter pylori*; recurrent aphthous stomatitis

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Introduction

Helicobacter pylori (HP) is a microaerophilic, Gram-negative bacterium, which causes chronic active gastritis and plays a primary role in the pathogenesis of both gastric and duodenal ulcer (Lambert *et al*, 1995).

Approximately 50–70% of HP strains produce two cytotoxins: (1) VacA, whose action is enhanced by acid pH causing vacuolation and degeneration of the epithelial cells of gastric mucosa and (2) CagA, a surface protein, which constitutes the main infectious element of HP and is associated with greater HP colonization of gastroduodenal mucosa and advanced gastroduodenal disease. It possibly causes a specific T-lymphocyte response and could be a potential candidate antigen for vaccine development (Peterson and Graham, 1998; Shimoyama and Crabtree, 1998).

Helicobacter pylori has been detected in gastric secretions, faeces, saliva in the dental plaque of healthy individuals and also in patients with upper digestive system diseases (Lambert *et al*, 1995; Nguyen *et al*, 1995).

The presence of HP has been correlated with ulcerative and neoplastic diseases of the digestive system, such as, gastric lymphoma of low malignancy (MALT) and gastric cancer (De Giacomo *et al*, 1990; Mollenkopf *et al*, 1990; Strauss *et al*, 1990; Sierra *et al*, 1992; Sipponen *et al*, 1992; Blaser *et al*, 1993; Farinati *et al*, 1993; Lambert *et al*, 1995; Matsukura *et al*, 1995; Niemann *et al*, 1997; Siman *et al*, 1997; Sozzi *et al*, 1998).

These observations led us to investigate the potential role of HP in recurrent aphthous stomatitis (RAS). The data regarding the potential relationship between RAS

and HP infection are limited and conflicting (Porter and Scully, 1991; Birek *et al*, 1999; Riggio *et al*, 2000; Shimoyama *et al*, 2000; Iamaroon *et al*, 2003; Richter *et al*, 2003; Victoria *et al*, 2003).

The aim of the present study was to investigate the role of HP in the aetiology of RAS and the effect of eradication treatment of the microorganism on the clinical course of the disease.

Materials and methods

We studied 48 patients all with minor recurrent aphthous ulceration referred to the Oral Medicine Clinic. There were 26 women and 22 men, of mean age 41.3 + 2.44 years. The clinical criteria for detection were the same in all patients and were evaluated according the number, duration and the frequency of the lesions. The diagnosis of RAS was based on clinical criteria that were proposed by Porter and Scully (1991) and Scully *et al* (1996).

Clinical assessment of RAS was based on a scale as follow: 0, no symptoms; 1, mild symptoms; 2, moderate symptoms; 3, severe symptoms. Mild symptoms were characterized the presence of one to two ulcers, whose duration was 4–7 days and their frequency of recurrence was every 2–3 months. As moderate were regarded the existence of two to five lesions with 10–15 days duration and frequency every 1 month. Severe were characterized by the presence of more than five lesions, with more than 15 days duration and continuous ulceration.

All patients were healthy and did not manifest any systemic disease. Patients on anti-secretory medication (H2 histamine antagonists and proton pump inhibitors), antibiotics and bismuth derivatives were excluded from the study. Thirty-four patients were HP positive and the rest 14 were negative. The latter were used in our study as the control group.

The diagnosis of HP infection was based on the detection of specific IgG and IgA antibodies using the enzyme-linked immunoabsorbent assay (ELISA) technique in the serum and the saliva of the patients. The presence of IgG, IgA, and CagA antibodies to HP was determined by using a commercial ELISA kit (Sorin Biomedica Diagnostics, Kasel, Germany).

The cut off values used in the study were 10 iu ml^{-1} for HP IgG, 15 iu ml^{-1} for HP IgA and 10 iu ml^{-1} for antiCagA.

Ninety-two well microtitre plates coated with HP antigens were used for the detection of anti- HP IgG & IgA in serum and saliva samples of all patients.

The HP carriers were given triple eradication treatment of omeprazole 20 mg b.i.d. plus clarithromycin 500 mg b.i.d. and amoxicillin 1 g b.i.d. for 7 days. After a 2-month period a ^{13}C-Urea INFAI test (Cufa Institute, Bochum, Germany) was performed to assess the HP infection status among the patients and was repeated at 1 and 2 years after eradication therapy (Labenz and Bosch (1994).

The follow-up period lasted 12 months with monthly visits to the clinic and included a detailed clinical assessment with particular attention to the number, the size and the recurrence rate of lesions.

Symptom scores were comparatively assessed in HP positive patients before and after eradication treatment. Clinical comparisons were also made between the HP carrier group before treatment and patients who were HP negative from the beginning, and lastly between the group of patients who became negative after treatment and the group who were HP negative from the beginning.

Statistical analysis of the results was performed using the χ^2 test and the unpaired *t*-test. χ^2 test was used for categorical analysis of positive and negative to HP patients, while *t*-unpaired test was used for the comparison of antibody values before and after treatment.

Results

On the basis of specific IgG and IgA anti-HP antibodies and anti-CagA protein, antibodies to HP were determined in the serum and the saliva of 48 patients with RAS. Thirty-four of 48 (70.8%) patients were HP positive.

As shown in Table 1, 29 patients had IgG antibodies in the serum and 12 patients had IgA antibodies. Saliva anti-HP IgA antibodies showed greater sensitivity compared with that of IgG (16 patients *vs* five patients).

In 18 patients CagA was present in serum and in three of them in saliva as well. Table 1 also presents the title range for IgG, IgA and anti-CagA antibodies in serum

Table 1 Range of the antibody titre to *Helicobacter pylori* in the serum and saliva in patients with recurrent aphthous stomatitis (RAS) and their relation with the intensity of the disease

	IgG (Cut off value = 10 iu ml^{-1})	*IgA (Cut off value = 15 iu ml^{-1})*	*CagA (Cut off value = 10 iu ml^{-1})*
Serum			
Mean value	55 ± 61 iu ml^{-1}	95 ± 37 iu ml^{-1}	47.1 ± 39.4 iu ml^{-1}
Range	14.3–137 iu ml^{-1}	18–95 iu ml^{-1}	10–110 iu ml^{-1}
No. of positive patients	29	12	18
Saliva			
Mean value	13 ± 12 iu ml^{-1}	49.7 ± 29.7 iu ml^{-1}	43.33 ± 5.77 iu ml^{-1}
Range	10–16 iu ml^{-1}	16–80 iu ml^{-1}	10–50 iu ml^{-1}
No. of positive patients	5	16	3
Intensity of RAS symptoms	Mild	Moderate	Severe

Table 2 The therapeutic effects of eradication therapy in 24 of 34 patients with recurrent aphthous stomatitis who became *Helicobacter pylori* negative

Complete cure	*Significant improvement*[a]	*Moderate improvement*[b]
Therapeutic response 7/24 (29.5%)	8/24 (33.3%)	9/24 (37.5%)

[a]When improved by two stages from severe to mild.
[b]When improved by one stage from severe to moderate or moderate to mild

and saliva of patients with RAS and their relationship with the severity of symptoms.

After the administration of an appropriate HP eradication treatment, 15 of 24 patients became negative and showed remarkable improvement with regard to both the recurrence intervals, which were longer, and the symptom intensity of the disease. Seven of 24 patients, showed complete cure, becoming free of symptoms (29.5%), while eight patients (33.3%) demonstrated a decrease in both the frequency of the recurrence (approximately every 2 months) and symptom intensity. This improved clinical status remained stable during the whole follow-up period. However, nine out of 24 of those who had become HP negative showed moderate clinical improvement (Table 2).

Statistical evaluation before the beginning of treatment showed that patients with HP infection suffered from more severe symptoms with regard to frequency of recurrence intervals and symptom intensity compared with initially HP negative patients ($P < 0.05$) (Table 3).

It is noteworthy that after eradication treatment, the recurrence intervals in patients who had become negative were significantly longer compared with those before treatment ($P < 0.001$), a finding that suggests the beneficial effect of the treatment in the decrease of recurrences of the disease (Table 3). The intensity of the symptoms was also decreased.

Furthermore, another important observation was that the clinical status of HP positive patients, who became negative after the eradication treatment, was almost comparable with that of the 14 out the 48 patients who had been HP negative from the beginning of the study ($P > 0.05$) (Table 3).

Table 3 Comparison of patient groups with recurrent aphthous stomatitis by *Helicobacter pylori* status before and after treatment

Patients	*Interval ulcer free (in days)*	*P*-value
Positive before treatment ($n = 34$)	31.32 ± 38.62	< 0.001
Becoming negative after treatment ($n = 24$)	85.26 ± 60.22	
Positive before treatment ($n = 34$)	31.32 ± 38.62	< 0.05
Negative from the beginning ($n = 14$)	140.71 ± 188.69	
Becoming negative after treatment ($n = 24$)	85.26 ± 60.22	> 0.05
Negative from the beginning ($n = 14$)	140.71 ± 188.69	

Discussion

The aetiology of RAS remains unclear. Auto-immune mechanisms or genetic factors have been implicated in its pathogenesis. In 5% of the cases it may be associated with gastrointestinal diseases (Kayavis *et al*, 1987; Albanidou-Farmaki *et al*, 1988).

Clinical observations have demonstrated a relationship between a number of gastrointestinal diseases and aphthae. In particular, oral ulceration (aphthae) has been described in patients with malabsorption syndrome or idiopathic steatorrhea, ulcerative colitis and Crohn's disease (O Mahony *et al*, 1985; Velso and Salciro, 1987; Majorana *et al*, 1992).

Helicobacter pylori has been established to play an important role in the evolution and development of the ulcerative diseases of the upper digestive system (Blazer, 1990; NIH Consensus Conference, 1994).

This study explores the possibility of HP colonization in GI tracts of patients with RAS. In the majority of our patients with oral aphthous ulcers the presence of specific HP IgG antibodies in the serum was at a percentage of 70.8%, IgA in saliva up to 33.8%, and anti-CagA antibodies in the serum at a percentage of 37.5%. Our observations are consistent with the results of other researchers in previous studies (Birek *et al*, 1999; Riggio *et al*, 2000; Shimoyama *et al*, 2000).

Our findings also suggest that serological tests for the detection of IgG antibodies against HP are useful in the diagnosis of HP infection, while they seem to show a relation with the severity of the disease (Table 1). IgA antibodies seem to have a limited presentation in serum, but their presence in saliva is increased, a finding that is in accord with earlier studies (Sugiyama *et al*, 1995).

Our results clearly show that patients with recurrent oral ulceration appear to suffer from active HP infection in a high percentage up to (70.8%) of cases. This percentage is significantly higher; almost double than found that in healthy adults of the same age (40–50 years old) in Greece (40–50%) (Apostolopoulos *et al*, 2002). Moreover, it is remarkable that the eradication treatment of HP in patients with oral ulceration resulted in complete remission or considerable improvement of symptoms in a high percentage of patients (62.5%). This favourable result, which was maintained throughout the whole period of follow-up (12 months after the eradication treatment), was found only in patients who became HP negative after the eradication treatment. These findings may imply a possible aetiological correlation between HP infection and RAS.

The exact mechanism by which HP induces tissue injury is not clear. Immune-mediated mechanisms induced by HP have been the subject of intense investigation. HP strains have the ability to stimulate cytokine production, particularly IL-8 and to induce secretion of lymphocyte chemotactic factors with the

formation of particular T lymphocyte subpopulations. Infiltrating neutrophils can be activated by the bacterium or its extracts to produce reactive oxygen metabolites (hydrogen peroxide and hypochlorous acid) all of which are cytotoxic (Birek *et al*, 1999).

The complex aetiology of RAS including genetic, environmental, hormonal, infectious and immunologic factors has been recognized since a long time. There is a increasing evidence that focal T-cell mediated immunity (delayed type of hypersensitivity reaction or a cytotoxic response) is the mechanism ultimately responsible for tissue destruction. It is not known what particular exogenous or endogenous factors (allergens or autoantibodies) residing in the oral epithelium might trigger the immune response. However it is conceivable that in susceptible individuals mucosal changes may develop which permit the adherence of HP and subsequent production of autoantibodies to epitopes shared by oral epithelial cells and microorganisms. Furthermore, cytokine production, over expression of lymphocyte adhesion molecules and the recruitment of specific subsets of T-lymphocytes, have all been shown to play a role in oral aphthous stomatitis as they have also in HP-associated gastritis. Thus, it appears likely that HP acts at least as a co-factor in the pathogenesis of recurrent oral stomatitis, especially in individuals sensitized through gastric colonization and mucosal attachment (Bodger *et al*, 1997; Gasbarrini *et al*, 1999).

It is also possible that the patients with HP related gastritis may have some iron and other micronutritient deficiencies that may predispose them to RAS. Thus treatment for HP may eliminate these co-factors in disease causation.

The present study does not prove an aetiological relationship between HP infection and RAS. However, it does show a strong correlation in a large subgroup (62.5%) of patients, who demonstrated a complete or considerable remission of symptoms after HP eradication treatment. This result is important, in view of the recurrent symptomatology of this disease, which negatively affects the quality of life of these patients (Llewellyn and Warnakulasuriya, 2003), and who need a range of therapeutic interventions in order to achieve clinical improvement.

In conclusion, the results of this study support the idea of a potential relationship between RAS and HP, which might or might not be aetiological. This association is seen at least in a large subgroup of patients. However, this issue still remains open and needs to be further investigated and confirmed by other controlled clinical studies.

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Molecular Analysis of B-Cell Clonality in *Helicobacter Pylori* Gastritis

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The aim of our study was to identify PCR-detectable clonal B-cell population in *Helicobacter pylori* gastritis and assess their relation to the Wotherspoon-Isaacson (W-I) grade for gastric lymphoid infiltrates. Amplified DNA was obtained from thirty four *H. pylori* positive gastritis dyspeptic patients and thirty four *H. pylori* negative matched controls. Clonal bands were observed in 6 (2/17 W-I Grade 1, 2/13 W-I Grade 2, and 2/4 W-I Grade 3 lesions) and polyclonal smears in 24 cases (15 W-I Grade 1, 7 W-I Grade 2, and 2 W-I Grade 3). Four additional W-I Grade 2 samples with clonal bands were associated with background polyclonal smear and were not reproducible. Clonal bands were not recorded in controls. B-cell clonality was not related to W-I grades. We conclude that certain *H. pylori* positive gastritis patients show PCR-detectable monoclonality, which is independent of the W-I grade of gastritis and cannot be taken as evidence of an existing neoplastic lesion.

KEY WORDS: *Helicobacter pylori*; gastritis; B-cell; clonality; PCR; MALT.

Helicobacter pylori infection of the gastric mucosa causes immunological response that leads to chronic gastritis with lymphoid follicles within the stomach. These follicles are composed of reactive T cells and activated B cells. A clonal expansion of these B-cells represents the basis of Mucosa- Associated Lymphoid Tissue (MALT) B-cell lymphoma (1).

The gold standard for diagnosis of MALT lymphoma is histopathology (2). However, superficial and small numbers of endoscopic gastric biopsies are limiting factors in histological evaluation (3). Polymerase chain reaction (PCR) based assays (3, 4) to detect the expansion of monoclonal B cells have also been used to corroborate the diagnosis. Clonal B-cell populations have been detected in patients with *H. pylori* gastritis, with uncertain clinical significance (3) and they also persist for many years after cure of *H. pylori* infection and complete endoscopic and histological remission of the lymphoma (5). The aim of our study was to identify the existence of clonal B-cell populations in *H. pylori* gastritis and to explore the correlation of B-cell clonality with the degree of lymphoid infiltrates of the gastric mucosa.

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PATIENTS AND METHODS

Patients. We prospectively evaluated consecutive patients with dyspepsia with upper gastrointestinal endoscopy. Patients after *H. pylori* eradication therapy or on proton pump inhibitors or on systematic use of non-steroidal anti-inflammatory drugs were excluded. Out of 200 consecutive patients, organic lesions (peptic ulcers or tumours) were identified in 51 patients during endoscopy. These patients were excluded from further evaluation. From the rest of the patients ($n = 149$), two random endoscopic biopsies were obtained from the antrum, two from the corpus and one from the incisura of the gastric mucosa. Informed consent was obtained from study participants to agree in using part of the bioptic material for the purpose of our investigation. The study was conducted according to the declaration of Helsinki for human rights.

Histology—Immunohistochemistry. Formalin fixed and paraffin embedded gastric biopsies were used. Morphological evaluation was based on 4 μm thick Haematoxylin & Eosin (H&E) sections. Biopsies were classified using the updated Sydney classification (6), for the degree of activity and severity of inflammation, glandular atrophy and intestinal metaplasia. *Helicobacter pylori* infection of the gastric mucosa was assessed by a modified Giemsa method and confirmed immunohistochemically with the anti-*Helicobacter pylori* monoclonal antibody (Dako, Glostrup, Denmark). Patients were classified *H. pylori* negative if the above tests were both negative, and *H. pylori* positive if both tests were positive. All samples were classified according to the Wotherspoon-Isaacson (W-I) system for lymphoid infiltrates within the gastric mucosa (2).

Molecular Analysis. DNA was extracted and purified according to standard protocols from 10 μm sections. In order to obtain the highest DNA yield, the DNA extraction consisted of deparaffinization by xylene-ethanol, protein digestion with Proteinase K (Sigma, USA) at 50°C overnight and no further purification steps.

The integrity of DNA was assessed by amplifying sequences of b-globin gene with nested PCR yielding a product of 272 bp of globin gene.

Two protocols, each involving two rounds of PCR, were used for amplification of the Ig heavy chain gene (7–9) using a consensus third framework region primer (FRIIIA) and a consensus second framework region primer (FRIIA) (Thermo Electron Corporation, Germany).

In each case the first round of amplification used primer FRIIIA (5'-ACACGGC-[C/T][G/C]TGTATTACTGT-3') and FRIIA (5'-TGG[A/G]TCCG[C/A]CAG[G/C]C-[T/C][T/C]CNGG-3') plus a down stream consensus primer directing to the joining region (LJH: 5'-TGAGGAGACGGTGACC-3'). The second used the same upstream primers in conjunction with the inner down stream primer VLJH (5'-GTGCCAGGG-TNCCTTGGCCCCAG-3'). In both rounds the total volume of the reaction mixture was 50 μL; it contained 250–500 ng of tested DNA, Tris-HCl (pH 9.0), 50 mM KCL, 0.1% Triton-X, 200 μM of each dNTP, 1.5 mM $MgCl_2$ and 1.25 U-Taq DNA Polymerase (Promega Corporation, USA). The first round PCR contained 250–500 ng of tested DNA and the second-round PCR contained one microlitre of first-round PCR product. Thirty first round and twenty second round cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C were carried out on a thermo-cycler (Biometra, Germany). In each round an initial denaturation step (5 min at 94°C) and a primer extension step (10 min at 72°C) concluded the PCR reaction.

Ten microliters of PCR amplified product were run on 4% agarose gel or on 10% polyacrilamide gel for 40–60 min at 130 V. Gels were stained with ethidium bromide, viewed under UV light and documented using a video camera.

Clonality was established by the presence of discrete bands ranging in size from 80 to 150 bp for the FRIIIA primer and 240–280 bp for FRIIA primer, whereas polyclonal rearrangements appeared as a diffuse smear pattern or multiple faint indistinct bands.

During PCR, strict precautions were taken to minimize cross contamination of samples. In addition, two controls were used: a positive (known lymphoma case previously shown to have a clonal band by PCR) and a negative without DNA to check for the presence of any contaminant.

All tests were run in duplicate, in order to test the reproducibility of the method and to detect any false positive result, that is a second PCR from the same DNA sample. Furthermore, in cases with clonal bands (with or without a background smear) a second PCR from DNA from deeper sections was also performed.

Statistical Analysis

Data were entered in the statistical package SPSS 10.0.0 for Windows, (SPSS Inc., Chicago, IL). To compare differences between groups we used non-parametric test, as appropriate. A p-value of less than 0.05 indicated statistical significance.

RESULTS

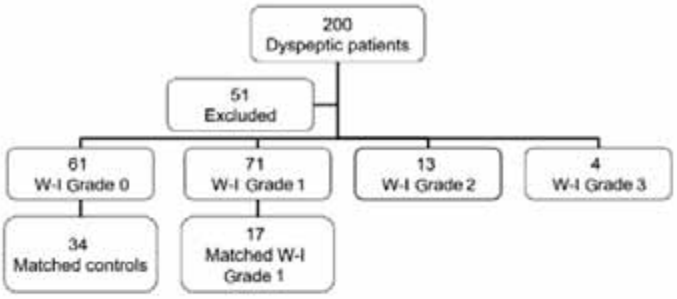
The histological characteristics of our patients are shown in Figure 1. Out of 149 dyspeptic patients, 88 (59%) were *H. pylori* positive and 61 (41%) were *H. pylori* negative. Out of 88 *H. pylori* positive patients, we identified seventeen (nine males, 25–79 years old) Grades 2 and 3 according to the W-I system for gastric lymphoid infiltrates patients. Out of 71 W-I Grade 1 *H. pylori* positive patients, we studied 17 (nine males, 19–80 years old) patients, gender and age matched to the group of W-I Grades 2 and 3 subjects. Thirty-four (18 males, 18–69-years old) dyspeptic subjects with normal histology, gender, and age matched to the 34 *H. pylori* positive patients, served as controls. No case of W-I Grade 4 or MALT lymphoma was identified.

PCR was negative for VDJ IgH rearrangements in the gastric mucosa of all *H. pylori* negative controls. Clonal bands were detected in 6 samples of *H. pylori* positive dyspeptic patients (2/17 W-I Grade 1, 2/13 W-I Grade 2 and 2/4 W-I Grade 3 lesions), as shown in Figure 2. Polyclonal smears were observed in 24 cases (15 W-I Grade 1, 7 W-I Grade 2 and 2 W-I Grade 3). Four additional W-I Grade 2 samples with clonal bands were associated with background polyclonal smear and were not reproducible when duplicate PCR tests were run.

The presence of B-cell monoclonality did not differ significantly among W-I Grades 1, 2, and 3 (Pearson's chi-square test, two-sided, exact, $p = 0.2$). Moreover, in the presence of lymphoid follicles (W-I Grades 2 and 3) monoclonality was equally distributed among the two groups (Fisher's Exact test two-sided, exact, $p = 0.66$).

DISCUSSION

The distinction between the neoplastic or the reactive nature of a gastric lymphoid infiltrate may be difficult in some cases, even with adequate morphological and immunohistochemical approach. Clonality analysis of lymphoid cells using the polymerase chain reaction (PCR) to



W-I Grade: 0=Normal histology, 1=Chronic active gastritis (CAG), 2=CAG with florid lymphoid follicles, 3=Suspicious lymphoid infiltrates in lamina propria, probably reactive

Fig 1. Flow diagram of the patients studied and classification of histology according to the W-I system.

amplify the VDJ junctional region of the immunoglobulin heavy chain (IgH) gene seems to be a useful aid with an overall sensitivity and specificity of 88% and 100% respectively (9).

The detection of B-cell clonality by PCR in *H. pylori* gastritis varies among studies between 0 and 85%. This variation can be explained by the different scoring criteria for monoclonality, differences in methodology or histology and case selection bias, which might be a key factor (3, 4, 10–22). In our prospective case-control study, we examined gastric mucosa tissue from dyspeptic patients without any endoscopic (mass or ulcer lesion) or histological evidence for the presence of MALT lymphoma. Our data show that monoclonal cells can be found in gastric biopsies in patients with *H. pylori* gastritis irrespectively of the presence of lymphoid follicles.

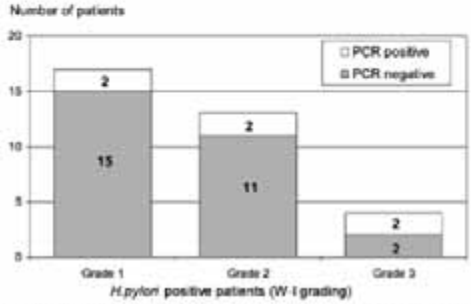


Fig 2. Distribution of "pure" clonal bands using strict criteria for monoclonality (constant, reproducible bands) in relation to the grade of lymphoid infiltrates (W-I grading system).

We showed an overall incidence of 23% of monoclonal B-cell populations in W-I Grades 2 and 3 lesions (16 and 50% respectively) and 12% in W-I Grade 1 lesions. No monoclonal band was detected in the gastric mucosa of the *H. pylori* negative controls. The low incidence of B-monoclonality in our W-I Grade 1 population is in accordance with previous publications (4, 21), although figures up to 25% have also been published (17). In cases with lymphoid infiltrate the reported percentage of monoclonality ranges from 24 to 50% (3, 4, 17, 22). Only Torlakovic et al. report 85% monoclonality for cases with *H. pylori* gastritis and lymphoid hyperplasia (14).

PCR can be routinely applied to paraffin-embedded endoscopic biopsies (3, 4, 8, 9, 20) but one must be aware of the method's limitations with respect to sample size. The sensitivity of the assay allows the detection of small imbalances in B cell populations, raising the possibility of false positive results. Thus, data from clonal analysis of small or lymphocyte poor samples must be evaluated critically. It is recommended that analysis should be run in parallel on at least two DNA samples and only reproducible bands present in more than one sample should be evaluated (3).

In order to obtain representative and adequate material for our PCR-analysis, an H&E section was examined prior to sections for DNA preparation to ensure that lymphoid tissue was still present. The DNA extraction method which was used gives excellent quality DNA extracted from formalin-fixed paraffin embedded tissues (23). Furthermore, the electrophoresis of at least two DNA samples from each gastric biopsy in clonal cases served as an internal control to avoid false positive results. Both samples should have given PCR products at the same level during electrophoresis, in order to be specific.

Regarding the primers selection, we used a FRIII primer because it has been proven that the highest, specific monoclonality rate (82% in B-non Hodgkin lymphoma and 0% in polyclonal gammopathy) is observed using CDR III region amplification (24).

In 2002, a multicentre study was carried out to demonstrate the heterogeneity of PCR assays. According to their results, fixation lowers the sensitivity of IgH PCR assays to detect clonal bands on formalin-fixed paraffin embedded tissues and the addition of FRIIA to the FRIIIA upstream primer increases the detection sensitivity from 57.3 to 73.6% (25). Since we used formalin-fixed paraffin embedded tissues in our study, we added the FRIIA primer.

Monoclonality was not associated with the degree of lymphoid infiltrates in our *H. pylori* positive samples assessed by the W-I scoring system. Miyamoto *et al.* showed that the incidence of monoclonal B-cell populations was significantly higher in *H. pylori* positive patients with follicular gastritis than in *H. pylori* positive controls (22). Wundisch *et al.* found that the detection of monoclonality was independent of the W-I grading system for W-I Grade 2 and 3 biopsy samples, while they detected a strong association of monoclonal B-cell population with the presence of lymphoid follicles when the compared W-I Grade 0 and 1 samples with the W-I Grade 2 and 3. If W-I Grade 0 cases are excluded, no relation between clonality and W-I score can be found (4). Moreover, the presence of lymphoid follicles in routine endoscopic biopsy specimens is a variable phenomenon, depending on a number of clinical, demographic and histological parameters (26).

We detected clonal B-cell bands against a background of polyclonal smear in four samples. The monoclonal band was not reproduced in any of these cases, when a PCR test was performed in a deeper section. Similar findings are seen in both reactive lymphoid lesions and lymphomas reflecting the background of non-neoplastic B-cell population (3, 14).

However, the presence of clonal B-cells could support a link between *H. pylori* gastritis and lymphoma or might represent a stage in the natural history of the disease, not necessarily meaning progression to malignancy. Both Saxena *et al.* (3) and Hsi *et al.* (11) did not find any relation between the presence of B-cell clonality and later evolution to lymphoma after follow-up periods of 21–44 and 59 months respectively. In contrast, another study showed that clonality was significantly more frequent in those gastritis lesions that later developed lymphoma (15) and there are documented cases of MALT lymphoma rose on gastritis through the development of B-cell clones (27). Moreover, a persistent monoclonality detected by PCR after complete histological remission of MALT lymphoma might indicate a population at risk for relapse of the tumor (5).

In conclusion, we showed that B-cell clonality could be detected in certain *H. pylori* gastritis dyspeptic patients. This finding was independent of the W-I grading for the lymphoid infiltrates. In the absence of any histological evidence of lymphoma, the clinical relevance of this finding remains questionable and open for further evaluation.

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Gastric Mucosa Epithelial Cell Kinetics Are Differentiated by Anatomic Site and *Helicobacter Pylori* Infection

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Changes in epithelial cell turnover related to *Helicobacter pylori* infection may contribute to gastric cancer development. The response of different anatomic sites of the gastric mucosa to *H. pylori* is not known. We studied apoptosis and cell proliferation at the grater and lesser curvature of the antrum and corpus, the fundus, and the cardia from 9 *H. pylori* gastritis patients and 11 *H. pylori*-negative controls with normal histology. Proliferation was highest at the major curve of the antrum and lowest at the fundus, and apoptosis was highest at the cardia and lowest at the major curve of the antrum in both *H. pylori* gastritis and normal mucosa. Proliferation was significantly higher at all anatomic sites, while apoptosis was significantly lower only at the major and lesser curve of the corpus in *H. pylori* gastritis compared with normal controls. Our data suggest that gastric mucosa epithelial cell kinetics is differentiated by the anatomic site and *H. pylori* infection.

KEY WORDS: gastric mucosa; apoptosis; cell proliferation; gastric cancer; *Helicobacter pylori*.

Gastric cancer evolution has been proposed as a multistep process ranging from chronic gastritis to invasive cancer (1), involving a deregulation of both cell proliferation and programmed cell death (2). Meta-analyses have shown that *Helicobacter pylori* colonization of the stomach is a risk factor for gastric cancer development, suggesting a relative risk of between 1.9 (95% CI, 1.3–2.8%) and 2.5 (95% CI, 1.9–3.4%) (3).

Alterations of epithelial cell turnover resulting from *H. pylori* infection may play a key role in carcinogenesis. Several studies have dedicated *H. pylori* infection as an inducer of apoptosis (4–8) and a stimulator of cell proliferation (7, 9–12). Furthermore, *H. pylori* gastritis predominantly affects the gastric antrum and corpus (13), and gastric cancer most often develops in these anatomic sites (14). However, the underlying mechanisms remain to be defined and the response of different anatomic sites of the gastric mucosa to *H. pylori* infection is not known. The aim of our study was to investigate the apoptotic and proliferation rates at different anatomic sites of the stomach possibly reflecting a different immune response and/or a mechanism by which *H. pylori* alters epithelial cell turnover and drives subsequently to the development of neoplasia.

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METHODS

Patients. Twenty nonulcer dyspepsia patients were studied. Patients who had *H. pylori* eradication therapy or were on proton pump inhibitors or on systematic use of nonsteroidal anti-inflammatory drugs were excluded. It is our standard practice to

take multiple random gastric mucosa biopsies from endoscopy-negative nonulcer dyspepsia patients. Informed consent was obtained from patients to agree in using part of the bioptic material for our investigation. The study was conducted according to the Helsinki Declaration for Human Rights. For the purpose of our study, we obtained gastric mucosa biopsies from six different anatomic sites in the stomach, namely, the major and lesser curve of the antrum and the corpus, the fundus, and the cardia.

Histology–Immunohistochemistry. Formalin-fixed and paraffin-embedded tissue sections of 4-μm thickness were made. They were classified using the Sydney classification (13), for the degree of activity and severity of inflammation, glandular atrophy, and intestinal metaplasia. *Helicobacter pylori* infection of the gastric mucosa was assessed by a modified Giemsa method and confirmed immunohistochemically with anti-*Helicobacter pylori* monoclonal antibody (DAKO, Glostrup, Denmark). Patients were classified as *H. pylori* negative if the above histology tests were both negative and as *H. pylori* positive if both tests were positive. Biopsies from *H. pylori*-negative dyspepsia patients with normal histology were used as normal controls.

Immunostaining was performed using the labeled streptavidin–biotin technique to detect Ki-67 antigen in proliferating cells and *H. pylori*. Tissues were incubated with monoclonal MIB-1 and anti-*Helicobacter pylori* monoclonal antibody (DAKO), respectively. Biotinylated antimouse IgG was used as a secondary antibody (DAKO), followed by peroxidase-conjugated streptavidin (DAKO).

Labeling of Apoptotic Cells by TUNEL Assay. Apoptotic cells were detected using the terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) method with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Intergen Company, Oxford, UK). Following permeabilization with proteinase K (20 μg/ml; Intergen Company) and blocking of endogenous peroxidase, the slides were incubated with working-strength TdT enzyme solution. Rinsing in a working-strength stop/wash buffer for 10 min terminated the reaction. The sections were incubated with anti-digoxigenin-peroxidase conjugated antibody. The peroxidase reaction was developed using a substrate with 3,3′-diaminbenzidine tetrachloride (0.25 mg dissolved in 1 ml of 0.02% hydrogen peroxide) and sections were lightly counterstained with hematoxylin.

Computer Image Analysis. The percentage of positively stained epithelial cells was determined by observer-interactive computerized image analysis (SAMBA microscopic image processor). Estimation of the standard error of the mean within 95% confidence limits required a maximum of at least randomly selected 15 high-power fields (HPF; X400-Zeiss microscope; analysis per area of approximately 110,000 μm^2). The immunostaining was analyzed as dark brown color with counterstained cells as false blue. Formal scoring (labeling index) for each antibody was then performed in one section for each paraffin block. Intraobserver variability was very low (<0.03%). The results are expressed as a percentage of the immunopositive nuclear surface in relation to the total nuclear surface of foveolar cells within the area of the tissue measured (labeling index), as previously described (15).

Statistical Analysis. Data were analyzed by the statistical package Statgraphics 4.0 (Manugistics Inc., Statistical Graphics Corp., Rockville, IL, USA). Numerical data were assessed by the nonparametric Mann–Whitney two-sided U test and the Kruskal–Wallis T test used for two- and multiple-sample comparison analysis, respectively. Regression analysis with the best fitting to our data curvilinear model was used to evaluate correlation of proliferation and apoptosis indexes in *H. pylori*-positive and *H. pylori*-negative controls. A P value of <0.05 indicated statistical significance.

Results in the text are presented as median with ranges. Data in figures are presented as box-and-whisker plots. The box includes 50% of the results falling between the 25th and the 75th percentile. The median value is represented as a horizontal line inside the box. Outliers, i.e., points more than 1.5 times the interquartile range from the end of the box, are shown as open squares.

RESULTS

Ten males and 10 females having a median age of 34 (range, 18 to 54) years were studied. All had endoscopy-negative nonulcer dyspepsia. Nine patients had *H. pylori* gastritis and 11 *H. pylori*-negative normal gastric histology (normal control group). *Helicobacter pylori*-positive patients were older (39.4 vs. 28.5; $P = 0.03$) than controls.

The median degree of the activity and severity of inflammation was 3 (range, 2–3) in the seven *H. pylori*-positive patients classified as having gastritis in all compartments of the stomach (pangastritis), while the median degree was 2 (range, 1–3) in the two patients with antral predominant gastritis. Only three of the *H. pylori*-positive patients showed atrophy (degree range, 2–3) and intestinal metaplasia (degree range, 1–3). All controls had normal histology.

Median proliferation indexes were significantly different among all six anatomic sites of the gastric mucosa studied, in both *H. pylori* gastritis ($T = 50$, $P < 0.001$) and normal mucosa ($T = 56$, $P < 0.001$), being highest at the major curve of the antrum and lowest at the fundus (Figure 1). Furthermore, *H. pylori*-infected mucosa had a significantly higher proliferation index at all anatomic sites compared with the mucosa of controls ($P < 0.001$).

Median apoptosis indexes were also significantly different among all six anatomic sites of the gastric mucosa studied, in both *H. pylori* gastritis ($T = 47$, $P < 0.001$) and controls ($T = 60$, $P < 0.001$), being lowest at the major curve of the antrum and highest at the cardia (Figure 2). However, *H. pylori*-infected compared with noninfected mucosa had a significantly lower apoptosis index only at the major and lesser curve of the corpus ($P = 0.03$).

Pooled data for all six anatomic sites showed that there was a significant correlation between proliferation and apoptosis indexes in both in *H. pylori* gastritis [proliferation = exp(2.63 + 2.04/apoptosis), $r^2 = 70.6$, $F_{1,52} = 125$, $P < 0.001$] and controls [proliferation = exp(2.34 + 1.65/apoptosis), $r^2 = 77.9$, $F_{1,54} = 225$, $P < 0.001$) (Figure 3). The regression lines of these two

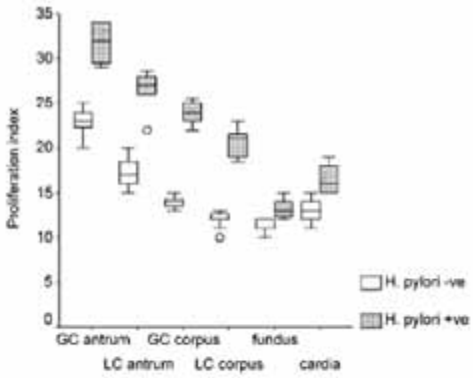

Fig 1. Comparison of proliferation indexes between *H. pylori*-positive patients ($n = 9$) and *H. pylori*-negative controls ($n = 11$). *Helicobacter pylori*-infected mucosa had a higher median index at all six anatomic sites of the gastric mucosa compared with normal mucosa ($P < 0.001$). GC, greater curvature; LC, lesser curvature.

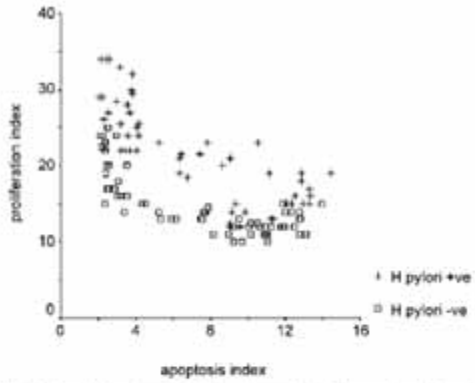

Fig 3. Correlation of proliferation and apoptosis indexes of pool data of six anatomic sites of the gastric mucosa in *H. pylori* gastritis and normal mucosa. There is a significant correlation both in *H. pylori* gastritis ($r^2 = 70.6$, $P < 0.001$) and normal mucosa ($r^2 = 77.9$, $P < 0.001$). The regression lines of these two correlations have significantly different slopes and intercepts ($P < 0.001$), probably indicating that normal and *H. pylori*-infected mucosa have a different cell turnover.

correlations have significantly different slopes and intercepts ($P < 0.001$).

DISCUSSION

Epidemiological studies suggest that gastric cancer predominantly affects the antrum and the corpus of the stomach, while relative and absolute increases in the incidence of proximal gastric cancer have been described recently (3, 14). Whether these indicate a different response of the gastric mucosa of antrum, corpus, fundus, or cardia to factors such as epithelial cell turnover and *Helicobacter pylori* infection is unknown.

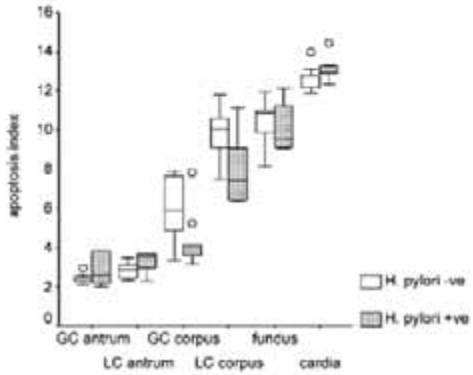

Fig 2. Comparison of apoptosis indexes between *H. pylori*-positive patients ($n = 9$) and *H. pylori*-negative controls ($n = 11$). *Helicobacter pylori*-infected mucosa had a higher median index only at the major and lesser curve of the corpus compared with normal mucosa ($P = 0.03$). GC, greater curvature; LC, lesser curvature.

Cell proliferation is essential in normal cell life, while apoptosis plays an opposite role in regulating cell populations. Changes of the balance in cell turnover may result in disturbance of tissue homeostasis and are associated with various cancers (16). These alterations have been described in gastric carcinogenesis and related to *H. pylori* infection (5–12, 16), but to our best knowledge, proliferation and epithelial cell apoptosis rates of different anatomic sites of the stomach have never been studied.

Our study is the first investigating six different sites of the gastric mucosa including the lesser and major curve of the antrum and of the corpus, the cardia, and the fundus. Apoptosis and cell proliferation in relation to *H. pylori* infection have been studied in the past, in human tissue samples from the gastric antrum or the antrum and the corpus of the stomach. However, these studies examined cell kinetics indistinguishably from the lesser or major curve and they do not provide any data from the fundus and cardia (4–12, 16–27). The specific finding of the present study is that proliferation and cellular apoptosis rates have a close relationship not only with *H. pylori* infection (Figure 3), but also with the different anatomic sites of the gastric mucosa (Figures 1 and 2). Our finding that the regression lines of the correlations of proliferation

and apoptosis are significantly different between normal gastric mucosa and *H. pylori*-infected mucosa cells probably indicates that normal and *H. pylori*-infected mucosa have a different cell turnover.

Furthermore, we have shown that proliferation is significantly higher in *H. pylori* gastritis compared with normal mucosa, the highest median value observed in the antral mucosa of the major curve. Apoptosis rate was statistically lower in both the major and the lesser curve of the corpus of *H. pylori* gastritis compared with normal controls.

Our results agree with studies showing that *H. pylori* colonization significantly increases cell proliferation rate (9–12, 16–19). In addition, we have shown that irrespective of the presence of *H. pylori* infection, cell proliferation is significantly different between the different anatomic sites of the gastric mucosa, being highest in the antrum and lowest in the fundus. Furthermore, our data show that the apoptotic rate is significantly lower in the major curve of the antrum and higher in the cardia, irrespective of the presence of *H. pylori*. This inverse relation between proliferation and apoptosis may be related to the preference of gastric cancer for distal parts of the stomach.

Apoptosis rate was significantly decreased in the corpus of the stomach in *H. pylori* gastritis, while a not statistically significant increase was observed in the antrum and in the cardia of the same patients compared with normal mucosa. Published data regarding gastric cell apoptosis and *H. pylori* infection are controversial. *Helicobacter pylori* has been associated with an increase in gastric epithelial apoptosis in most studies (4–8, 17, 18–26). However, in other studies, apoptosis was reported as unchanged (27) or even decreased (28) in the presence of *H. pylori*, and Moss *et al.* reported increased apoptotic rates only in the presence of cag+ *H. pylori* strains (17). Other investigators found a relatively higher proliferation rate in *H. pylori*-positive patients, within foci of intestinal metaplasia (18, 19), associated with a decreased apoptotic rate. This could contribute to *Helicobacter pylori*- associated gastric carcinogenesis. Due to the small number ($n = 9$) of *H. pylori*-positive patients, it was not possible to statistically correlate proliferation and apoptosis rate with the histologic features of the gastric mucosa.

Our data suggest that there is a disturbance between cell proliferation and apoptosis depending on both *Helicobacter pylori* status and the anatomic site of the gastric mucosa. Is this evidence of a relatively different effect of *Helicobacter* on the different combination of cells and glands throughout the different gastric compartments? The different environment in the antrum than in the corpus (29) may also play a role in the way *Helicobacter pylori* affects cell kinetics in the different anatomical sites of the gastric mucosa. It also seems possible that the differences among anatomical sites of the stomach may reflect the normal histology of the stomach, although the pathologists tried to include the same number of total foveolar cells per section in the measurements.

In conclusion, our study addresses for the first time apoptosis and cell proliferation in different anatomical sites of both *H. pylori* gastritis and normal gastric mucosa. The detected differences may play a role in the altered distribution of precancerous or cancerous neoplastic lesions in different sites of the stomach.

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Association between *Helicobacter pylori* infection and acute inflammatory demyelinating polyradiculoneuropathy

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The aim of this study was to investigate a possible association between *Helicobacter pylori* infection and acute inflammatory demyelinating polyradiculoneuropathy (AIDP). Of 17 consecutive patients with Guillain–Barré syndrome (GBS), 13 patients (six females; mean age 50 ± 24 years) with AIDP were investigated. Clinical status was evaluated according to Hughes' score, and electrophysiological tests were performed within 2 weeks from disease onset. *Helicobacter pylori* infection was detected histologically and serum *H. pylori*-specific IgG antibodies were analysed by ELISA. Twenty asymptomatic patients (12 females; mean age 63 ± 8 years), undergoing upper gastrointestinal endoscopy for investigation of mild iron deficiency anaemia, served as controls. *Helicobacter pylori* was found in 12 of 13 AIDP patients (92%), and in 10 of 20 controls (50%), ($P = 0.02$). Electrophysiological studies showed demyelination in all AIDP patients. High levels of anti-*H. pylori* IgG antibodies correlated with advanced clinical status. Five of seven AIDP patients with high levels of anti-*H. pylori* IgG antibodies had delayed F-wave latencies, indicating affection of proximal segments of peripheral nerves. *Helicobacter pylori* infection seems to be more frequent in AIDP patients. Anti-*H. pylori* titre might reflect advanced clinical status. Anti-*H. pylori* IgG antibodies are also associated with involvement of the proximal parts of peripheral nerves in AIDP.

Introduction

Guillain–Barré syndrome (GBS), an acute immune-mediated disease that targets the myelin and/or the axons of the peripheral nerves (Press *et al.*, 2001), is the foremost cause of acute, generalized, peripheral neuropathic weakness worldwide. In North America and Europe, acute inflammatory demyelinating polyradiculoneuropathy (AIDP) is the commonest observed subtype of GBS. Moreover, the commonest antecedent infection recognized before the onset of GBS is because of *Campylobacter jejuni* (Rees and Hughes, 1994), which has also been associated with more severe axonal degeneration in Western patients. *Helicobacter pylori*, colonizing the gastric mucosa of most humans worldwide, mainly affects older adults in the developed world, including Greece (The EUROGAST Study Group, 1993; Peterson and Graham, 1998). It is associated with various upper gastrointestinal diseases (Peterson and Graham, 1998) and extradigestive vascular conditions (Kountouras *et al.*, 2001, 2002). In particular, *H. pylori* is thought to be associated with the development of autoimmune sequelae observed in peripheral neuropathies (Kornberg and Pestronk, 1993; Chiba *et al.*, 1998) and GBS (Chiba *et al.*, 2002), where autoantibodies to specific neural targets impair native neural function and, furthermore, with autoimmune conditions like Sjögren's syndrome (Aragona *et al.*, 1999), and chronic open-angle glaucoma (Kountouras *et al.*, 2003a,b).

To date, limited data suggest a role for *H. pylori* as antecedent infection in AIDP in particular (Chiba *et al.*, 2002). Therefore, we conducted this study to investigate a possible association between *H. pylori* infection and AIDP.

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Patients and methods

Of 17 consecutive patients with GBS, fulfilling the diagnostic criteria for GBS (Asbury and Cornblath, 1990), 13 patients (six females; mean age of 50 ± 24 years, range 18–80 years) with AIDP, according to clinical and electrophysiological criteria

(Ho *et al.*, 1997) (group A), were studied. Hughes' functional grading scale score (Hughes *et al.*, 1978) was used to evaluate the patients' clinical status (grade 0 = healthy; grade 1 = minor signs or symptoms of neuropathy but capable of manual work; grade 2 = able to walk without support of a stick but incapable of manual work; grade 3 = able to walk with a stick, appliance, or support; grade 4 = confined to bed or chair bound; grade 5 = requiring assisted ventilation; and grade 6 = dead), performed by trained observers, blinded to *H. pylori* findings, daily during hospitalization up to day 30. All patients had a severe, monophasic illness. Within the first 4 days post-admission, all patients underwent the endoscopic procedure and serological and cerebrospinal fluid (CSF) measurements. Their initial clinical condition was well-retained, and, although seven patients were bedridden, all the patients were eating per os; therefore, no intubation or nasogastric tubes were necessary to be introduced. Electrophysiological tests were performed within 2 weeks from the onset of the disease. Nerve conduction studies of median, ulnar, common peroneal, and sural nerves were performed by using standard methods. The electrodiagnostic protocol for AIDP, as outlined previously (Albers *et al.*, 1985), was completed. Nerve conduction velocity, amplitude of the compound muscle action potential, F-wave response latency as well as distal motor latency were studied and used as electrophysiological diagnostic criteria for the diagnosis of AIDP (Ho *et al.*, 1997). Follow-up needle electromyography (EMG) was performed 5–6 weeks after the onset of the disease to distinguish reduced recruitment secondary to diffuse conduction block from axonal degeneration. Blood and CSF were examined for presence of viral, *Borrelia burgdorferi* and syphilis infections. All patients underwent serological test for mycoplasma.

The control subjects (group B) consisted of 20 asymptomatic patients (12 females; mean age 63 ± 8 years, range 44–70 years), who were undergoing upper gastrointestinal endoscopy for the investigation of mild iron deficiency anaemia, with normal results. None of the subjects had received any treatment, like $FeSO_4$, before the diagnosis.

All 33 patients (13 AIDP patients, 20 anaemic controls) underwent elective diagnostic upper gastrointestinal endoscopy after informed consent. Exclusion criteria have been previously reported (Kountouras *et al.*, 2002).

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.

Study design

Helicobacter pylori detection methods

Subjects reported at 9 AM after a 12-h fasting. Intravenous sedation with midazolam (3–10 mg) was given, and standard upper gastrointestinal endoscopy was performed with a forward viewing videoscope (Olympus CE 0197; Opto-Electronics Co. Ltd, Tokyo, Japan) to identify evidence of macroscopic abnormalities. Simultaneously, three biopsy specimens were obtained from the antral region within 2 cm of the pyloric ring and three from the corpus. One biopsy specimen from each site was used for rapid urease slide testing of *H. pylori* infection (CLOtest; Delta West, Draper, UT, USA), and the other two biopsy specimens were placed in 10% formalin and submitted for histological examination. Before endoscopy, venous blood was drawn from each patient for serological testing of *H. pylori* IgG antibodies. Sera were stored at −20°C for analysis of IgG antibodies against *H. pylori* as described previously (Kountouras, 1998; Kountouras *et al.*, 2002).

Biopsy urease test

Each biopsy specimen was placed in a tube containing 0.5 ml of 10% urea in deionised water to which had been added two drops of 1% phenol red as a pH indicator (CLOtest; Delta West). The biopsy specimen test was read as described previously (Kountouras *et al.*, 2002).

Histopathology

All specimens were stained with haematoxylin and eosin. For detection of *H. pylori* organisms Cresyl fast violet and/or Giemsa stains were preferred. Moreover, intestinal metaplasia was evaluated with Alcian blue stain. The same experienced pathologist (I.V.) assessed all specimens, being unaware of the other determinants of the *H. pylori* status.

Helicobacter pylori serology

Helicobacter pylori serology status was determined using a commercial enzyme-linked immunosorbent assay kit (Elias, Osceola, WI, USA), as described previously (Kountouras *et al.*, 2002).

Statistics

For comparison of the age (years) between AIDP patients and control subjects the Mann–Whitney *U*-test was used, whereas for gender the Fisher exact test was

applied. The latter test was also used to compare the prevalence of *H. pylori* infection between AIDP patients and control subjects. Odds ratios and 95% confidence intervals (CI) were also calculated between these two groups. Significance was set at $P < 0.05$.

Results

Mean age and sex ratios did not differ between AIDP patients and anaemic controls (Table 1). *Helicobacter pylori* infection was diagnosed in 12 of 13 AIDP patients (92%) and in 10 of 20 anaemic controls (50%), as confirmed by the presence of *H. pylori* bacteria histologically [$P = 0.02$; odds ratio 12.0, 95% confidence interval 1.7–86.0]. *Helicobacter pylori*-specific IgG antibodies were detected in serum by ELISA in 12 of 13 (92%) AIDP patients and in seven of 20 (35%) anaemic controls ($P = 0.001$).

Clinical status (Hughes' scale), days of progression, demographical and electrophysiological characteristics, as well as serum anti-*H. pylori* IgG levels in each patient are shown in Table 2. High levels of anti-*H. pylori* IgG antibodies usually correlated with a more advanced clinical status (grade 4 in Hughes' scale). Five of seven patients with high levels of anti-*H. pylori* IgG antibodies had delayed F-wave latencies, indicating affection of proximal segments of peripheral nerves. One patient (no. 7) was admitted to ICU on the 16th day post-admission because of respiratory complications.

Electrophysiological studies showed a pattern of demyelination in all patients with AIDP. More specifically, conduction studies showed reduction of conduction velocity (motor conduction velocity: 85 ± 5% of normal mean value; sensory conduction velocity 96 ± 4% of normal mean value), delay of F-response latency [98 ± 3% of the upper limit of normal value (ULN)], reduction of temporal dispersion (62 ± 6% of normal mean value), as well as prolonged distal latency (89 ± 6% of ULN). All values are expressed as mean ± SD. Sensory nerve action potential of sural nerve was normal. Conduction study results from individual nerves were expressed as percentage of normal mean value for each nerve and then average for motor and sensory nerve values. In addition, individual electrodiagnostic evaluation was reviewed to determine the specific criteria for demyelination. All AIDP patients had conduction abnormalities of at least two of the above-mentioned conduction parameters in two nerves or two abnormalities in three tested nerves (median, ulnar and peroneal nerves). Values of 2 SD above the mean were considered abnormal. Needle EMG findings were normal.

The detailed electrophysiological features of the patients are shown in Table 2. These results are the hallmarks showing that demyelination was the predominant type of peripheral nerve damage in our patients. From the above-mentioned conduction parameters, we observed that five of seven patients (no. 2, 3, 6, 8, 9, 11, and 12 patients in Table 2) with very high levels of anti-*H. pylori* IgG antibodies had delayed F-wave latencies, indicating the affection of proximal segments of peripheral nerves.

Discussion

Based on histological analysis for the documentation of *H. pylori* infection, the present study suggests an association between *H. pylori* infection and AIDP. In our Greek cohort, 12 of 13 patients with AIDP (92%) exhibited histologically proven *H. pylori* infection, whereas the rate of infection was significantly lower in the anaemic control group (50%). The prevalence of *H. pylori* infection in our control group is similar to that reported by other investigators when using serodiagnostic assays to evaluate Greek cohorts and other ethnic populations showing a frequency distribution of 34–62%.

Our study has relied upon histology for the documentation of *H. pylori* infection. Although culture is the theoretical gold standard for detection of the bacter-

Table 1 *Helicobacter pylori* positivity detected by ELISA in serum, and by histology in patients with AIDP and anaemic controls

	AIDP ($n = 13$)	Controls ($n = 20$)	Significance	
			P	Odds ratio (95%CI)
Age (years), mean (range)	50 ± 24 (18–80)	63 ± 8 (44–70)	NS	...*
Sex (men:women)	7:6	8:12	NS	...
Presence of *H. pylori* by ELISA (IgG > 10 U/ml)	12 (92%)	7 (35%)	0.001	22.3 (3.0–164.6)
Histological presence of *H. pylori*	12 (92%)	10 (50%)	0.02	12.0 (1.7–86.0)
Total *H. pylori*-positive cases	12 (92%)	10 (50%)	0.02	12.0 (1.7–86.0)

ELISA, enzyme-linked immunosorbent assay; AIDP, acute inflammatory demyelinating polyradiculoneuropathy.
*Ellipses indicate not applicable.

Table 2 Electrophysiological and clinical characteristics, and serum anti-*Helicobacter pylori* IgG levels in each patient with AIDP

Patient	Sex/age	Electrophysiological study				Serum anti-*H. pylori* IgG (U/ml)	Clinical status[e] (Hughes' scale)	Days after onset	Autonomic dysfunction
		CV[a]	FwL[b]	CAMP[c]	DL[d]				
1	M/18	↓			↓	5.1	2	13	
2	M/78		↓		↓	129	4	12	Tachycardia
3	M/57	↓	↓			72	1	14	
4	F/80	Normal				26	2	15	
5	F/21		↓			23.4	1	13	
6	F/72	↓	↓			113	4	15	
7	M/48	↓			↓	56	5	16	Respiratory symptoms (admission to ICU)
8	F/42	↓			↓	100	2	12	Fluctuating hypertension–hypotension
9	M/50		↓	↓		163	4	17	
10	F/76		↓		↓	32	4	18	
11	M/16	↓		↓		60	4	20	
12	F/61	↓	↓			76.8	4	17	
13	M/26	↓	↓			28.3	2	12	

[a]Conduction velocity; [b]F-response latency; [c]Amplitude of the compound muscle action potential; [d]Distal latency; [e]At the peak of the disease.

ium, it has been shown that there is an excellent correlation with histological identification (Weinstein, 1993). 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 (Weinstein, 1993; Peterson and Graham, 1998). However, an upper gastrointestinal endoscopy is required to obtain specimens for histology or culture of *H. pylori* and this method is costly and sometimes uncomfortable for patients. Therefore, for screening purposes and/or following up the efficacy of *H. pylori* treatment, non-invasive tests, such as a breath test or serological detection of IgG antibodies to *H. pylori* may be preferable.

Although our results did show a significant increase in serum IgG anti-*H. pylori* levels in AIDP patients compared with controls, the diagnostic significance of this test is limited because it does not discriminate between current and old infections (Meyer *et al.*, 1991) and it requires invasive sampling of blood (Kountouras *et al.*, 2001). Therefore, the endoscopic gastric mucosa histologic analysis seems to be the most reliable diagnostic procedure for *H. pylori* detection.

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

GBS is an acute inflammatory polyneuroradiculopathy, which is considered to be caused by autoimmune processes, triggered by a preceding bacterial or viral infection. Autoantibodies to specific neural targets have been found to impair native neural function. A number of single antigenic structures have been suggested to be targeted by the immune system. Although studies have shown that GBS is frequently preceded by acute infectious illness (Mishu and Blaser, 1993), the exact trigger of GBS is unknown and a specific immunological explanation has not been found. It is probably that immune responses directed towards the infecting organisms are involved in the pathogenesis of GBS by cross-reaction with neural tissues. The infecting organism induces humoural and cellular immune responses that, because of the sharing of homologous epitopes (molecular mimicry), cross-react with ganglioside surface components of peripheral nerves. Immune reactions against target epitopes in Schwann-cell surface membrane or myelin result in AIDP. Interestingly, molecular mimicry of host structures by the saccharide portion of lipopolysaccharides of the gastrointestinal pathogens *C. jejuni* and *H. pylori* are thought to be connected with the development of autoimmune sequelae observed in GBS (Mishu and Blaser, 1993).

Our results indicate that: (i) the serum high levels of anti-*H. pylori* IgG antibodies closely correlate with a more advanced clinical status, and (ii) elevations of anti-*H. pylori*-specific IgG antibodies are associated with involvement of the proximal parts of peripheral nerves in AIDP (as it was found in five AIDP patients). These re-

sults are in accordance with previous data demonstrating: (i) positive antibodies to VacA of *H. pylori* in the CSF in six of eight patients with AIDP, and (ii) delayed F-waves latencies in two of three patients with AIDP and positive antibodies to VacA of *H. pylori* in serum (Chiba *et al.*, 2002). Moreover, it has recently been reported that the target molecules of the specific antibody against VacA in the CSF obtained from patients with GBS are probably associated with some components of the peripheral nerve myelin, thereby suggesting a potential role in the immune responses of patients with the demyelinating form of GBS (Chiba *et al.*, 2003).

In view of the above-mentioned data, it is possible to suggest that the increased titre of anti-*H. pylori* IgG antibodies observed in the serum samples of AIDP patients may indirectly offer evidence for a role of *H. pylori* in the cascade of events of the damage of the myelin and/or the axons of the peripheral nerves. An interesting correlation is the observation that anti-*H. pylori* IgG antibody titre appeared to correlate with the severity of the clinical status and involvement of the proximal parts of peripheral nerves in AIDP patients. Nevertheless, future studies on larger AIDP cohorts are needed to support the hypothesis that the presence of IgG antibodies to *H. pylori* may adversely influence progression of AIDP neuropathy and whether there are changes in the titre of anti-*H. pylori* IgG antibodies during the follow-up visits, as well as the possibility that these changes may be related to the clinical status at the same time. If a causal link between *H. pylori* infection and AIDP is confirmed in the future, this may have a major impact on the pathophysiology and management of AIDP.

Competing interests

None declared.

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Gastroesophageal Reflux Disease and Helicobacter pylori: Lack of Influence of Infection on Oesophageal Manometric, 3- Hour Postprandial pHmetric and Endoscopic Findings

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Abstract

Aim. To investigate the relationship between Helicobacter pylori and gastro-oesophageal reflux disease according to manometric and pHmetric findings. **Method.** Fifty-nine consecutive patients with reflux symptoms and endoscopic evidence of mild oesophagitis, were recruited. Manometry and ambulatory pHmetry were performed in all patients, as well as the 3-hour postprandial pHmetry, as a more flexible and well tolerable test. **Results.** There were no significant differences between Helicobacter pylori positive and negative patients regarding age, sex ratio and endoscopic severity of oesophagitis. There was no difference in prevalence of abnormal oesophageal peristalsis between the two groups (Fisher's exact test, p=NS). Differences were also not found regarding lower oesophageal sphincter pressure between the two groups (mean P_{LOS} 12.86±4.39 mmHg and 13.1±4.61mmHg respectively; p=0.840). Finally, the mean values of DeMeester score were 60.38±48.04 and 67.64±51.04 respectively (p=0.576). **Conclusion.** Helicobacter pylori infection does not influence oesophageal peristalsis, the lower oesophageal sphincter pressure and the acidity of refluxates into the oesophageal lumen, in patients with established gastro-oesophageal reflux disease (esophagitis grade A and B).

Key words

Gastroesophageal reflux disease - Helicobacter pylori - 3-hour postprandial pH monitoring - oesophageal manometry - DeMeester score

Rezumat

Scop. Investigarea relaţiei dintre Helicobacter pylori şi esofagită pe baza modificărilor manometriei şi pH-metriei.

Metodă. Au fost studiaţi 59 pacienţi cu simptomatologie de reflux şi modificări endoscopice de esofagită uşoară. Pacienţii au fost examinaţi ambulator prin manometrie şi pH-metrie. A fost urmărită pHmetria la 3 ore postprandial, fiind un test mai flexibil şi mai bine tolerat. **Rezultate.** Nu au existat diferenţe semnificative între pacienţii Helicobacter pylori pozitiv şi cei Helicobacter pylori negativ privitor la vârstă, sex şi severitatea esofagitei determinată endoscopic. Prevalenţa peristaltismului esofagian anormal între cele două grupe nu a fost diferită (testul Fisher, p nesemnificativ). De asemenea nu au existat diferenţe între cele două grupuri în ceea ce priveşte presiunea sfincterului esofagian inferior (P_{SEI}=12.86± 4.61mmHg şi 13.1± 4.61mmHg respectiv ; p=0.840). Valorile scorului DeMeester au fost 60.38± 48.04 şi 67.64± 51.04 respectiv (p=0.576). **Concluzii.** Infecţia cu Helicobacter pylori nu influenţează severitatea esofagitei, peristaltica esofagiană, presiunea sfincterului esofagian inferior şi aciditatea refluxului esofagian la pacienţii cu boală de reflux gastro-esofagian.

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Introduction

Helicobacter pylori (Hp) has been demonstrated to be the aetiological factor in gastrointestinal diseases such as gastritis, peptic ulcer, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma. Despite the reports that reduced acid secretion related to Hp corpus gastritis might protect patients from reflux oesophagitis and the reports of reflux oesophagitis occurring after Hp eradication, the pathogenesis and possible relationship of Hp infection with reflux oesophagitis remains controversial (1).

Symptoms like heartburn and acid regurgitation are usually sufficient to verify the diagnosis of gastro-oesophageal reflux disease (GORD) (2). The test used as gold standard to confirm and quantify the gastro-oesophageal reflux, is ambulatory 24-hour oesophageal pH monitoring. A limitation of 24-hour pH monitoring is low tolerability (3). Moreover, patients cannot keep the probe in

for 24 hours (4) and a shorter pH monitoring may be beneficial on that aspect.

We evaluated oesophageal function between Hp positive and Hp negative Greek patients with endoscopically established GORD. The 3-hour postprandial oesophageal pH monitoring was introduced as a more flexible and well tolerable test to quantify GORD in this group of patients. The aim of this study was to investigate the relationship between Hp infection and reflux oesophagitis according to manometric and pH monitoring findings.

Material and methods

Patients

Fifty-nine consecutive adult patients (31 males and 28 females) with weekly reflux symptoms for at least one year and endoscopic evidence of Grade A or B erosive oesophagitis according to Los Angeles classification (5) were recruited in this study.

Exclusion criteria included suspected or confirmed malignant disease, past gastric or oesophageal surgery, more severe grade of oesophagitis, previous Hp eradication therapy, primary oesophageal motility disorders, anticoagulant therapy, hiatus hernia (independent factor contributing to GORD), pregnancy or lactation and age <16 years. Patients with any condition likely to result in poor compliance were also excluded from the study. All patients had stopped acid suppression therapy (four days beforehand for those taking antacids, one month beforehand for those using H_2-receptor antagonists or proton pump inhibitors), and underwent a four-week washout period during which medications that affect motility were tapered.

Standard upper gastrointestinal endoscopy (Fujinon 250 HR endoscope) was performed at 9 a.m., after a 12 hour fast, in order to evaluate presence of oesophagitis. Reflux oesophagitis was graded A (leastsevere) to D (most severe), according to the Los Angeles classification. Biopsies were obtained and processed for histopathologic study (two samples from both antrum and body) [by Giemsa staining] and rapid urease test (two samples from both antrum and body) [CLO test].

Oesophageal manometry and 3-hour ambulatory postprandial oesophageal pH monitoring was performed in all patients after obtaining their informed consent for the study.

Manometry and 3-hour postprandial pH-monitoring study

All patients performed oesophageal manometry and 3-hour postprandial oesophageal pH monitoring within three days after endoscopy. After an overnight fast oesophageal manometry was performed using 8 channels, low compliance, pneumohydraulic perfused manometric assembly without sleeve sensor (manometric pump-model PIP-4-8SS Mui Scientific). The manometric assembly was passed transnasally and the position of the lower esophageal sphincter (LOS) was determined using the station pull-through technique at 0.5 cm intervals (6). Manometric examination of the LOS also served as a guide to correct placement of the pH catheter.

After removal of the manometric catheter, a monocrystalline antimony pH catheter was passed transnasally and the electrode was positioned 5 cm above the proximal margin of the LOS. The electrode was calibrated in buffers with pH 4 and pH 7 before each study (pHmetry UPS 2020, MMS- Medical Measurement Systems BV). The 3-hour ambulatory postprandial oesophageal pH monitoring study was then carried out. Patients were encouraged to avoid coffee, alcohol, fruit juices, smoking and antacids and otherwise instructed to take meals and work according to their daily routine (7). Data acquisition was performed using a portable solid-state data logger. The start of a reflux episode was defined as an esophageal pH below the threshold of 4.0, and the end as an increase above pH of 4.0. Parameters of esophageal acid exposure included the DeMeester score and the esophageal acid exposure time (percentage of time esophageal pH <4%). In our study, reflux was defined and quantified using the DeMeester score (8). Semi-automated analysis was performed with the aid of commercially available software. A single investigator (JM) performed all analyses. Abnormal gastro-oesophageal reflux, or a positive test was defined as DeMeester score above 14.72.

DeMeester score, which was the base of the correlation between the subjects, is a complex index that takes into account the % of total time with pH<4, pH<4 in upright position, pH<4 in supine position, the number of reflux episodes with intra-oesophageal pH<4, the number of reflux episodes with intra-oesophageal pH<4 with duration over 5 minutes and the reflux episode with the greatest duration in minutes. DeMeester score is a complex index that takes into account all the above parameters for the eligible duration of the study (7).

The gold standard to confirm and quantify the gastro-oesophageal reflux, is ambulatory 24-hour oesophageal pH monitoring. A limitation of this study is low tolerability and a shorter pH monitoring may be beneficial on that aspect. We decided to perform the 3-hour oesophageal pH monitoring to quantify the gastro-esophageal reflux disease, a shorter test sufficient to confirm and quantify the gastrocosphageal reflux disease with the same sensitivity and specificity to 24-hour oesophageal pH monitoring (4).

Statistical analysis

The statistical analysis was performed using the SPSS for Windows package (version 11.0, SPSS, Chicago, IL). Data are presented as means±SD, means with 95% CI or median values with 5th and 95th percentiles when appropriate.

Results

Thirty patients (15 males and 15 females; mean age 45.10±12.29 years) were Hp negative and 29 patients (16 males and 13 females; mean age 38.93±12.55 years) were

Hp positive. There were no significant differences between the two groups regarding age, sex ratio and endoscopic severity of GORD (Table I).

Table I Baseline demographic, endoscopic, manometric and pH monitoring data for Hp positive and Hp negative patients

	Hp positive	Hp negative
No. of patients	29	30
Age, years mean±SD (range)	38.93±12.55 (21-65)	45.1±12.29 (24-67)
Males/females	16/13	15/15
Oesophagitis grade A/B	17/12	16/14
LOS pressure (P_{LOS}) mmHg mean (95% CI)	12.86 (11.18-14.53)	13.10 (11.37-14.82)
DeMeester score mean (95% CI)	60.38 (42.1-78.65)	67.64 (48.58-86.70)

Abnormal baseline oesophageal manometry (abnormal contraction duration, wave amplitude, and wave propagation) was observed in 13 Hp negative (43.3%) and 13 Hp positive (44.8%) patients. There was no difference in prevalence of abnormal oesophageal peristalsis between the two groups (Fisher's exact test). We also found no difference regarding lower oesophageal sphincter pressure (PLOS) between infected and non-infected patients (mean P_{LOS} 12.86±4.39 mmHg and 13.1±4.61mmHg respectively; p= 0.840).

Abnormal oesophageal pH monitoring (DeMeester score above 14.7) was observed in 84.7% of the patients. The proportion of abnormal oesophageal pH monitoring was 82.8% and 86.7% in infected and non-infected patients respectively (non significant). Therefore, Hp infection did not correlate with an abnormal oesophageal pH monitoring (odds ratio, 0.74; 95% CI, 0.18-3).

Mean values of DeMeester score were 60.38±48.04 and 67.64±51.04 for infected and non-infected patients, respectively (independent Student's t-test, p=0.576).

Discussion

In this study, we compared the manometric and pHmetric characteristics of Hp positive and Hp negative patients with established mild reflux oesophagitis in order to evaluate the possible influence of infection on these parameters. Although 24-hour oesophageal pH monitoring is the gold standard method for studying GORD, this is difficult to perform in a large number of patients due to poor compliance. We used the 3-hour postprandial oesophageal pH monitoring in this study, as more flexible, well tolerable and equally sensitive method (4). The possible relationship of Hp infection and GORD remains controversial despite much published data. Some studies found no difference in the prevalence of Hp infection between patients with GORD and healthy individuals (6,9) while others have shown a significantly lower prevalence in patients with oesophagitis (10).

There are several proposed mechanisms through which Hp can contribute to the pathogenesis of GORD. There is the concept that the gastric inflammation of the cardia may lower the threshold for transient relaxation of LOS by altering the sensitivity of vagal sensory receptors (11). Hp gastritis is accompanied by release of nitric oxide, cytokines and prostaglandins (12). This can also sensitise afferent nerves and reduce LOS pressure (13) and promote damage to the adjacent oesophageal mucosa. There is good evidence to indicate that the excessive production of prostaglandins in reflux oesophagitis drives a vicious cycle of LOS dysfunction, reflux, mucosal inflammation, aggravated LOS dysfunction and further reflux (14). On the other hand, it has been proposed that predominantly antral Hp gastritis is associated with an augmented gastrin release. At least in some patients with GORD it is possible that increased acidity along with a higher volume of gastric juice may aggravate reflux disease (15). About 50% of the patients with GORD have evidence of delayed gastric emptying. Antral gastritis may interfere with gastric emptying (16,17). Some studies suggest that Hp positive dyspeptic subjects have abnormalities of gastric motor function (18) but data on this subject are quite controversial (16,19,20). Direct damage on oesophageal mucosa could also be caused by bacterial factors such as ammonia derivates, cytotoxin and phospholipase (17). Although Moayyedi P et al. showed that H. pylori eradication therapy does not seem to influence relapse rates in GERD patients (21).

There are several proposed mechanisms by which the bacterium might protect against GORD. Hp releases substances that inhibit gastric acid secretion (22,23). Hp generates large amounts of ammonia (24) which has a neutralizing effect at gastric pH decreasing the corrosive potential of the gastric juice refluxing into the oesophagus. On the other hand, ammonia leads to a protective adaptation of gastric mucosa in rats (25). As ammonia appears in the gastric juice refluxing into the oesophageal lumen, it is conceivable that ammonia can also lead to a protective adaptation of the oesophageal mucosa. Severe corpus gastritis is associated with lower acid output. This effect is probably mediated by cytokines such as interleukin-1, by nitric oxide and a loss of M3 receptors (26,27). Moreover, Hp gastritis may progress to multifocal atrophic gastritis with the destruction of gastric glands and hypochlorhydria (28). In Hp positive patients with reduced acid output as a consequence of corpus gastritis the infection is associated with raised serum gastrin concentrations. Although controversial, gastrin, even in physiological concentrations, may increase the LOS pressure and prevent the gastroesophageal reflux (29). The topography of H. pylori gastritis could be the crucial point. Antrum predominant gastritis aggravates reflux disease whereas pangastritis or corpus predominant gastritis protects the oesophagus against damage by gastric juice.

There have been several studies, using oesophageal pH monitoring and manometry to delineate the relationship between Hp infection and GORD. Wu et al. studied patients with erosive oesophagitis and controls without reflux disease. There was no difference in the oesophageal acid exposure or the severity of oesophagitis between Hp positive and Hp negative patients (30). Hp infected patients had decreased amplitude of distal peristalsis and lower basal pressure at the LOS. They concluded that failed oesophageal peristalsis was significantly more prevalent in Hp positive patients. Our group found no difference in oesophageal peristalsis between Hp positive and Hp negative patients and no significant difference of the lower oesophageal sphincter pressure (P_{LOS}). Feng et al. investigated 20 patients who underwent 24-hour ambulatory oesophageal pH monitoring to confirm the diagnosis of GORD (31). Their LOS pressure was measured by oesophageal manometry. Fasting serum gastrin was determined by radioimmunoassay in 13 patients. They were divided in two groups according to Hp status. There were no significant differences in LOS Pressure and DeMeester score between the two groups. The findings of this study (31) are similar to ours. Gisbert et al. evaluated the relationship between Hp infection and the presence of oesophagitis on endoscopy and abnormalities on 24-hour oesophageal pH monitoring. They concluded that Hp infection was not associated with GORD (32). Tefera et al. estimated the effect of Hp eradication on oesophageal acid exposure. PH studies were performed before and 12 weeks after eradication therapy in 25 subjects (33). They found no change in total time of pH<4 in patients after eradication and concluded that Hp infection does not influence GORD. Finally, Fallone et al in a recent study demonstrates, using excellent GORD quantifying measures including validated symptom severity scores, endoscopy, and 24-hr pHmetry, that no significant differences exist in clinical or laboratory-related GORD manifestations between Hp-infected and non-infected GORD patients (34).

Current evidence-based recommendations suggest that Hp should not be treated with the intent to either improve reflux symptoms or prevent the development of reflux complications (35). However, if patients are to receive long-term acid suppressive therapy, they should be tested for Hp and treated if positive, to reduce the risk of PPI induced atrophic gastritis (36). According to our findings, Hp infection does not influence the severity of oesophagitis (in patients with mild esophagitis), the oesophageal peristalsis and the lower oesophageal sphincter pressure as measured by oesophageal manometry and the acidity of refluxates into the oesophageal lumen as measured by 3-hour ambulatory postprandial oesophageal pH monitoring.

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ORIGINAL ARTICLE

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High prevalence of *Helicobacter pylori* infection and monoclonal gammopathy of undetermined significance in patients with chronic idiopathic neutropenia

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Abstract The prevalence of *Helicobacter pylori* infection was evaluated in 120 patients with chronic idiopathic neutropenia (CIN), 8 patients with monoclonal gammopathy of undetermined significance (MGUS) associated with CIN, and 74 age- and sex-matched normal volunteers, all derived from the same geographical area. The purpose of the study was to investigate the possible causal relationships of *H. pylori* infection with the development of MGUS in CIN patients. We found that the prevalence of *H. pylori* infection was elevated to 69.2% in the group of CIN patients, 100% in the group of patients with CIN-associated MGUS, and 32.4% in the group of control subjects. No statistically significant difference, however, was found in the prevalence of *H. pylori* infection between CIN patients with concomitant MGUS and CIN patients without MGUS, no resolution of the gammopathy after eradication of the bacterium, no significant rise in the titers of serum anti-*H. pylori* antibodies, and no formation of an abnormal precipitation line in immunoelectrophoresis using a saline extract of NCTC11367 *H. pylori* reference strain as antigen. We concluded that there is no evidence that *H. pylori* infection is the cause of MGUS in CIN patients.

Keywords Chronic idiopathic neutropenia (CIN) · Monoclonal gammopathy of undetermined significance (MGUS) · *Helicobacter pylori* infection

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Introduction

Monoclonal gammopathy of undetermined significance (MGUS) is a widely used term indicating the presence of a monoclonal spike (M component) in protein electrophoresis not associated with any lymphoplasmacytic neoplasm. The condition is more frequent in older age groups and may be transformed to multiple myeloma or to a related malignancy at a rate of 1% per year [3, 4]. Its cause is unknown, but a recent study has suggested that MGUS may be occasionally due to *Helicobacter pylori* infection since eradication of the bacterium with antibiotics has resulted in normalization of the electrophorogram in about 28% of a series of MGUS patients [5]. However, Rajkumar et al. [14] did not find any significant difference between patients with MGUS and normal controls in *H. pylori* seroprevalence nor did they demonstrate resolution of the gammopathy after *H. pylori* eradication.

Studies in our department have shown that chronic idiopathic neutropenia (CIN), a granulocytic disorder mainly due to impaired neutrophil production in the bone marrow and/or enhanced neutrophil extravasation in the periphery [7, 9], is associated with increased prevalence of both MGUS [8] and *H. pylori* infection (unpublished observations). The relationship, if any, between *H. pylori* infection and CIN-associated MGUS is unknown. The current study was undertaken to investigate the prevalence of *H. pylori* infection in a relatively large number of CIN patients and to search for possible causal effects of *H. pylori* infection in the development of CIN-associated MGUS.

Patients, materials, and methods

Patients

Among 214 CIN patients registered in our Neutropenia Unit, MGUS was diagnosed in 8 patients, suggesting a proportion of 3.74%. Data from six of these patients have already been presented elsewhere [8]. The addition of two new cases herein did not change the overall prevalence of the disorder within the CIN patient population. Some clinical and laboratory characteristics of these patients are shown in Table 1.

A total of 120 CIN patients, 24 men aged 29–75 years (median: 54.5 years) and 96 women aged 15–76 years (median: 52.5 years), were evaluated for *H. pylori* infection. All patients fulfilled the diagnostic criteria of CIN applied in our department as previously detailed [9, 10]. The duration of the disease in CIN patients ranged from 4 to 168 months with a median of 72 months.

As controls we evaluated 74 healthy volunteers, 16 men aged 32–63 years (median: 47.5 years) and 58 women aged 29–72 years (median: 50.0 years). No statistically significant differences were found between CIN patients and control subjects for age and sex distribution as judged by the χ^2 test. The study was approved by the Ethics Committee of the hospital and informed consent was obtained from all subjects studied.

Biochemical parameters

Serum and urine proteins, protein electrophoresis, and serum immunoglobulin levels were evaluated in the Laboratory of Biochemistry of the hospital employing the respective conventional methods. The type of M component was determined with immunofixation using specific rabbit polyclonal anti-human IgG, IgA, and IgM antibodies (Paragon, Bacacos, Athens). The agar gel technique of immunoelectrophoresis was used to evaluate the antibody content of the M components against a sonicated saline extract of cultured *H. pylori* bacteria (NCTC11637 reference strain).

Detection of *H. pylori* infection and *H. pylori* eradication

Diagnosis of *H. pylori* infection was based on the positivity of at least two of five diagnostic tests applied, i.e., ^{13}C-urease breath test, *Campylobacter*-like organism (CLO) test, histologic detection of the bacterium in gastric mucosa biopsies, and increased titers of serum anti-*H. pylori* IgG or IgA antibodies (above 30 U/ml) (Pyloriset EIA-G III and EIA-A III, respectively, Orion Diagnostica, Espoo, Finland). The therapeutic regimen applied for eradication of the bacterium included clarithromycin (1 g/day), amoxicillin (2 g/day), and omeprazole (40 mg/day) for 10 days. Omeprazole (20 mg/day) was also administered for 20 additional days.

Statistical analysis

Homogeneity of the populations studied was tested with the χ^2 test. Numerical data were analyzed using the Mann–Whitney nonparametric U test. A probability equal to or lower than 5% was considered as statistically significant.

Results

H. pylori infection was diagnosed in 83 of 120 CIN patients studied, suggesting a proportion of 69.2%, and in 24 of 74 healthy controls, suggesting a proportion of 32.4%. The difference between these two proportions is highly significant ($p<0.0001$, χ^2 test) indicating that the prevalence of *H. pylori* infection is significantly increased in patients with CIN.

All eight CIN patients with concomitant MGUS enrolled in this study were positive for *H. pylori* infection. However, comparing the prevalence of *H. pylori* infection in patients with CIN-associated MGUS (100%) with the prevalence of 69.2% found in CIN patients without MGUS, no statistically significant difference could be documented, suggesting that the prevalence of *H. pylori* infection is not higher in patients with CIN-associated MGUS than in CIN patients without MGUS.

Table 1 Some clinical and laboratory data of the CIN patients with concomitant MGUS. *Dur* duration (months from the initial diagnosis), *PMN* neutrophils, *M comp* M component, *Igs* immunoglobulins, *BM* bone marrow, *BJ* Bence Jones, *Neg* negative

Patient ID	Age/sex	Dur (months)	PMN (/μl)	M comp type	Serum Igs			BM plasma cells (%)	Urine BJ
					IgG	IgA	IgM		
055	56/M	31	1623	IgGλ	1039	247	95	1.2	Neg
061	60/M	71	1776	IgGκ	1540	371	42	0.9	Neg
085	53/F	34	1458	IgAκ	654	335	60	1.3	Neg
104	72/F	43	1764	IgGκ	2055	348	163	2.6	Neg
134	67/M	151	1292	IgGκ	1618	308	102	3.6	Neg
187	51/F	96	845	IgGκ	2254	224	75	0.8	Neg
291	46/F	186	1364	IgGκ	1980	287	102	2.7	Neg
309	60/F	18	1720	IgGκ	1751	206	50	1.8	Neg

All CIN patients with concomitant MGUS had increased titers of serum anti-*H. pylori* IgG antibodies (above 30 U/ml). The observed values ranged from 38 to 161 U/ml with a median of 104 U/ml and a mean of 104±44 U/ml. These values did not differ significantly from those of 180±222 U/ml found in the group of CIN patients without MGUS. Similarly, no significant difference was found in the mean titer of serum anti-*H. pylori* IgA antibodies between patients with CIN-associated MGUS and CIN patients without MGUS.

Immunoelectrophoresis was performed in four of eight CIN patients with concomitant MGUS. Serum containing the M component was tested for abnormal precipitation line formation in the agar gel against a saline extract of *H. pylori* cultured bacteria. In no patient was abnormal precipitation line formation detected, suggesting that the bulk amount of the serum M component did not contain specific antibodies against *H. pylori*.

Two of our patients (patients 134 and 291) were hospitalized for severe respiratory infection and were treated with a variety of antibiotics including amoxicillin. In both patients, eradication of *H. pylori* was assumed from the normalization of the titers of serum anti-*H. pylori* antibodies tested 7 and 12 months later, respectively. No resolution of the gammopathy or reduction in the size of the M component was documented in these patients. In contrast, patient 291 developed overt multiple myeloma 16 years after the initial diagnosis of CIN with concomitant MGUS, while patient 134 died from prostatic cancer after 12 years of follow-up.

With the exception of one patient (patient 104) who died from an unrelated cause and another patient (patient 055) who refused any therapeutic intervention, the remaining patients received the above-mentioned eradication regimen. Eradication of the bacterium was assumed from the negative ^{13}C-urease breath test performed 4 or more months after the completion of treatment. In no patient was normalization of the electrophoretic pattern observed.

Discussion

According to the data presented herein, the prevalence of *H. pylori* infection in patients with CIN is significantly elevated compared to age- and sex-matched normal controls derived from the same geographical area as the patients, namely, the island of Crete. The cause of such a difference is unknown. One can hypothesize that *H. pylori* infection might be a causal agent for CIN. This possibility, however, cannot be supported by the available data on the pathophysiology of CIN [7, 10]. Another possibility is that CIN might pave the way for *H. pylori* infection. It is evident that additional studies are needed to characterize the possible relationship, if any, between *H. pylori* infection and CIN.

H. pylori has been recognized as the most common cause of chronic gastritis [1] and has been considered as a putative causal agent of gastric ulcer [2], gastric adenocarcinoma [11, 13, 15], and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [12]. It has been shown that the bacterium releases antigenic material capable of stimulating lymphoplasmacytic clones to produce initially a polyclonal and subsequently a monoclonal immune response [6]. On the basis of these assumptions, one could accept that *H. pylori* infection may be involved in the pathogenesis of MGUS in CIN patients. However, such a relationship could not be demonstrated in the current study. The titers of serum anti-*H. pylori* antibodies in the patients were not those expected from the relatively large amounts of the M component in the serum. On the other hand, no formation of an abnormal precipitation line was seen in immunoelectrophoresis.

It has been reported that M components in patients with MGUS may be reduced or even disappear with normalization of the electrophorogram following a successful *H. pylori* eradication [5]. In our patients with CIN-associated MGUS, no resolution or significant reduction of the gammopathy was documented after *H. pylori* eradication. Interestingly, no correction of neutropenia was noted in the CIN patients studied before and after *H. pylori* eradication (data not shown).

In conclusion, the prevalence of *H. pylori* infection in CIN patients is significantly increased. All CIN patients with concomitant MGUS studied herein were *H. pylori* positive, but a causal relationship between MGUS and *H. pylori* infection could not be demonstrated. No significant difference was found in the prevalence of *H. pylori* infection between CIN patients with and without MGUS, no resolution of the gammopathy after eradication of the bacterium was seen, no significant rise in serum anti-*H. pylori* antibodies was noted, and no abnormal precipitation line was detected in immunoelectrophoresis. On the basis of these findings, we concluded that there is no evidence that *H. pylori* infection is the cause of MGUS in CIN patients, although such a possibility cannot be entirely ruled out.

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Helicobacter pylori seroprevalence in patients with chronic obstructive pulmonary disease

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Summary An increased seroprevalence of *Helicobacter pylori* (*H. pylori*) and especially of the high virulent cytotoxin-associated gene-A (CagA) positive strains has been found in several extragastroduodenal pathologies, characterized by activation of inflammatory mediators. Moreover, it has been reported that the risk of chronic bronchitis may be increased in *H. pylori* infected patients. The aim of the present study was to assess the seroprevalence of *H. pylori* and in particular of CagA-positive virulent strains in patients with chronic obstructive pulmonary disease (COPD). We evaluated 126 COPD patients (88 males and 38 females, aged 61.3 ± 8.1 years) and 126, age and sex-matched, control subjects. All subjects enrolled underwent an enzyme-linked immunosorbent assay (ELISA) IgG serologic test for *H. pylori* and CagA protein. The prevalence of *H. pylori* infection in patients and controls was 77.8% and 54.7%, respectively ($P<0.001$) and that of CagA-positive *H. pylori* infection was 53.9% and 29.3%, respectively ($P<0.001$). Moreover, COPD patients had a significantly increased mean serum concentration of both anti-*H. pylori* IgG (118.3 ± 24.4 vs. 61.9 ± 12.9 U/ml, $P<0.001$) and anti-CagA IgG antibodies (33.8 ± 3.4 vs. 19.0 ± 1.5 U/ml, $P<0.001$). Finally, no statistically significant difference, as regards the spirometric values, was detected between *H. pylori* infected COPD patients and uninfected ones. In conclusion, *H. pylori* infection may be associated with COPD. Further studies should be undertaken to clarify the potential underlying pathogenetic mechanisms.

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Introduction

Helicobacter pylori (*H. pylori*) is a slow-growing, microaerophilic, gram-negative bacterium which colonizes gastric mucosa and elicits both inflammatory and immune lifelong responses, with release of various bacterial and host-dependent cytotoxic substances.[1] This bacterium seems to have a causative role in the development of chronic gastritis,[2] peptic ulcer disease,[3] low-grade B-cell lymphoma of gastric mucosa-associated-lymphoid-tissue (MALT-lymphoma)[4] and gastric cancer.[5] Recent studies suggest that *H. pylori* infection might, also, be associated with several extragastroduodenal pathologies characterized by activation of inflammatory mediators and/or induction of autoimmunity.[6–8] Therefore, increased *H. pylori* seroprevalence has been found in ischemic heart disease,[9] rosacea[10] and active bronchiectasis.[11] In patients with ischemic heart disease, an extremely high prevalence of the cytotoxin-associated gene-A (CagA) positive virulent strain of *H. pylori* has also been reported.[12] The CagA-positive are those strains that induce increased local and systemic, humoral and cellular inflammatory response.[13]

It is well known that the prevalence of chronic obstructive pulmonary disease (COPD) in peptic ulcer patients is increased two-to-three fold, compared with findings in ulcer-free controls.[14–16] The major factor underlying this association seems to be the impact of cigarette smoking on both diseases. However, in 1998, a pilot study in a small number of Italian patients showed that *H. pylori* infection, per se, might be related to an high risk of developing chronic bronchitis.[17] More recently, an epidemiological study in Danish adults suggested that chronic bronchitis might be more prevalent in *H. pylori* IgG-seropositive women than in uninfected ones.[18] These observations prompted us to perform a case-control study in a cohort of Greek patients with chronic bronchitis. We found extremely high *H. pylori* seropositivity.[19] However, the prevalence of *H. pylori* and especially of CagA-positive strains (that induce increased inflammatory response and have been associated with other extraintestinal disorders), in COPD patients remains still unknown.

Therefore, the aim of the present study was to assess the seroprevalence of *H. pylori* and in particular of CagA-positive virulent strains in a cohort of COPD patients and control subjects (outpatients with a variety of respiratory diseases). Moreover, we evaluated the association between *H. pylori* serological status and spirometric values in patients with COPD.

Methods

Patients selection

The present study was conducted at the 9th Department of Pulmonary Medicine, "Sotiria" Chest Diseases Hospital (Athens, Greece). The local ethics committee approved the study and written informed consent was obtained from each participant. Following a predefined protocol, between June 1, 2002 and October 31, 2003, 178 consecutive patients with COPD, diagnosed according to the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) guidelines, were recruited from the outpatient clinics. Briefly, COPD was diagnosed as "the presence of a postbronchodilator FEV_1 <80% of the predicted value in combination with an FEV_1/FVC <70% in any patient who has symptoms of cough, sputum production, or dyspnea and/or a history of exposure to risk factors for the disease".[20] Exclusion criteria were: (i) an exacerbation of COPD in the preceding month, as in those cases pulmonary function does not represent baseline levels, (ii) prior *H. pylori* eradication therapy, (iii) consumption of acid-suppressive drugs or antibiotics in the preceding 6 months and (iv) a history of vagotomy or operation on the upper gastrointestinal tract. A total of 52 patients were excluded. Therefore, 126 patients were eligible for analysis.

Control subjects selection

Controls were selected randomly from 204 consecutive subjects with other pulmonary disorders attending the outpatient clinics during the period of study (bronchial asthma, respiratory infections, lung cancer and sarcoidosis). Briefly, bronchial asthma was diagnosed as the "presence of symptoms of episodic wheezing, cough and shortness of breath responding to bronchodilators and reversible airflow obstruction documented in at least one previous pulmonary function study" Exclusion criteria for controls were: (i) a known history of COPD, (ii) a known history of gastrointestinal tract pathology including *H. pylori* infection and (iii) consumption of acid-suppressive drugs or antibiotics in the preceding 6 months. Finally, we selected 126 controls from among 204 subjects (60 with respiratory infections, 38 with lung cancer, 22 with asthma and 6 with sarcoidosis). Control subjects were matched with the COPD patients for sex, age (within 2 years) and socioeconomic status. Social class classification was based on the current

occupation according to the classification system of the United Kingdom Registrar General. Assignment to class group is determined as follows: social class I: skilled professionals, social class II: intermediate manual workers, social class III: skilled manual workers, social class IV: partly skilled manual workers and social class V: unskilled manual workers. All unemployed housewives were classified according to their husbands' occupation.

Lung function—COPD severity

In all COPD patients a complete medical history was taken and a physical examination was performed. Moreover, in all cases postbronchodilation spirometric values (FEV_1, FVC, FEV_1/FVC) were measured. The best value of three maneuvers was expressed as a percentage of the predicted value. Finally, classification of COPD severity was performed according to GOLD guidelines.[20] Briefly, three stages of COPD according to disease severity were recognized:

(I) Stage I (mild COPD) was characterized by mild airflow limitation (FEV_1/FVC $<70\%$ and $FEV_1 > 80\%$ predicted).
(II) Stage II (moderate COPD) was characterized by worsening airflow limitation ($30\% < FEV_1 < 80\%$ predicted) and
(III) Stage III (severe COPD) was characterized by severe airflow limitation ($FEV_1 < 30\%$ predicted) or the presence of respiratory failure or clinical signs of right heart failure.

Serological parameters

All subjects enrolled (COPD patients and controls) underwent an enzyme-linked immunosorbent assay (ELISA) IgG serologic test for *H. pylori* and CagA protein detection (HEL-P test, Park Co, Athens, Greece), in accordance with the manufacturer's guidelines. The specificity and sensitivity of the serology test, validated in our local population, were 95% and 85%, respectively.

All results were analyzed simultaneously by technicians who were unaware of whether the sample belonged to cases or controls. A positive, borderline and negative result was assigned when the concentration of IgG antibodies against *H. pylori* was greater than 25, between 20 and 25 and less than 20 U/ml, respectively. Moreover, when the concentration of IgG CagA antibodies was greater than 7.5 U/ml, between 5.5 and 7.5 U/ml and less than 5.5 U/ml the result was considered as positive, borderline and negative, respectively. Borderline results were omitted from further analysis.

Statistical analysis

Results are expressed as mean $\pm$ one standard deviation ($\pm$ SD). Significance of difference between groups was assessed by unpaired Student's *t*-test for continuous variables and χ^2-test for proportions. The statistical analysis was performed using the SPSS program (SPSS Inc, IL, USA) and *P*-values were two-tailed analyzed. *P*-values of less than 0.05 were considered statistically significant.

Results

The demographic data of both patients and controls are shown in Table 1.

There was no significant difference in age or sex distribution between the two groups. Table 1 shows also the spirometric values of patients with COPD. Among the COPD patients, 35 (27.7%) had mild disease (Stage I according to GOLD classification), 68 (53.9%) had moderate disease (Stage II) and 23 (18.4%) had severe COPD (Stage III).

Table 2 shows the analysis of the serological parameters. Both anti-*H. pylori* IgG seropositivity and anti-CagA IgG seropositivity were significantly higher in COPD patients than in control subjects. Moreover, COPD patients had a significantly increased mean serum concentration of both anti-*H. pylori* IgG and anti-CagA IgG antibodies.

The distribution of COPD patients according to COPD severity was as follows: (i) Stage I (mild COPD): 35 patients (27.7%), (ii) Stage II (moderate COPD): 68 patients (53.9%) and (iii) Stage III (severe COPD): 23 patients (18.4%). The spirometric values of COPD patients in relation with *H. pylori* infection

Table 1 Demographic data of COPD patients and controls and spirometric values of COPD patients.

Parameters	COPD patients ($n = 126$)	Controls ($n = 126$)	*P*-value
Age (yr)	61.3 ± 8.1	59.0 ± 7.3	ns*
Male sex (%)	69.8	69.8	ns*
FEV_1†	61.9 ± 18.5		
FEV_1/FVC†	63.2 ± 4.8		

*Not significant.
†Expressed as percentages of the predicted values.

Table 2 Serological parameters in COPD patients and control subjects.

Parameters	COPD patients ($n = 126$)	Control subjects ($n = 126$)	*P*-value
Anti-*H. pylori* IgG seropositivity (%)	77.8	54.7	<0.001
Anti-*H. pylori* IgG level (U/ml)	118.3±24.4	61.9±12.9	<0.001
Anti-CagA IgG seropositivity (%)	53.9	29.3	<0.001
Anti-CagA IgG level (U/ml)	33.8±3.4	19.0±1.5	<0.001

Table 3 The spirometric values of COPD patients in relation with *H. pylori* infection.

Parameters	*H. pylori* positive ($n = 98$)	*H. pylori* negative ($n = 28$)	*P*-value
FEV_1*	61.5±18.9	63.5±17.9	ns†
FEV_1/FVC*	63.0±4.9	63.9±4.6	ns†

*Expressed as percentages of the predicted values.
†Not significant.

are shown in Table 3. No statistically significant difference, as regards the values, was detected between *H. pylori* infected COPD patients and uninfected ones. Finally, the spirometric values did not differ significantly between COPD patients infected with CagA-positive strains (FEV_1: 59.1±19.7, FEV_1/FVC: 65.4±16.6) and those infected with CagA-negative strains (FEV_1: 62.4±5.1, FEV_1/FVC: 64.3±4.3, *P*: not significant).

Discussion

Data in the literature on the relationship between *H. pylori* infection and chronic obstructive pulmonary disease (COPD) are poor. COPD had been associated with gastroduodenal ulcer many years before the identification of *H. pylori* infection as a cause of peptic ulcer disease. Three epidemiological studies, carried out between 1968 and 1986, showed that the prevalence of COPD in peptic ulcer patients was increased two-to-three fold compared with that in ulcer-free controls.[14–16] Moreover, a follow-up study demonstrated that COPD was a major cause of death among patients with peptic ulcer disease.[21] The reported association between these two diseases was, originally, attributed to the known role of cigarette smoking as an independent factor in both ulcerogenesis and development of COPD.[22] However, two recent studies showed that a subpopulation of COPD patients, those with chronic bronchitis, might also have an increased prevalence of *H. pylori* infection.[17,19]

The present study is the first focused on the seroprevalence of *H. pylori* and in particular of CagA-positive virulent strains in a large population of patients with COPD. Patients who were at risk for COPD (STAGE 0 according to GOLD) were not included in our study as those patients might have only limited differences as regards lung function with controls, a fact that might represent a potential study limitation. According to our results, both anti-*H. pylori* and anti-CagA seropositivity were significantly higher in COPD patients than in control subjects. The socioeconomic status, which is related with both *H. Pylori* infection and risk of COPD, is similar in the two groups. Tobacco use could be another confounding factor. Cigarette smoking is the most important etiologic factor of COPD. However, data on the relationship between *H. pylori* infection and smoking habits are controversial. The prevalence of *H. pylori* infection in smokers has been variously reported as low,[23] normal,[24] and high.[25] In the present study, we did not match patients and control subjects for smoking habits. As the relation between smoking and *H. pylori* colonization of gastric mucosa has not been clarified yet, the possible impact of cigarette smoking on both COPD and *H. pylori* infection should be regarded as a potential study limitation.

The selection of control subjects should be considered as another study limitation. It has been suggested, in a few studies, that *H. pylori* might be increased in a variety of respiratory disorders including lung cancer, bronchiectasis and tuberculosis.[8] Therefore, the selection of patients with respiratory diseases as controls may have reduced

the difference in positive seroprevalence between the groups. However, the existed difference could not be attributed to this selection, as a low *H. pylori* seroprevalence in respiratory diseases has not been reported yet.[8]

The present study has not focused on the potential pathogenetic mechanisms underlying the association between *H. pylori* infection and COPD. This association might reflect either susceptibility induced by common factors or a kind of causal relationship between these diseases. As far as we know, there are no common factors implicated in the susceptibility to both COPD and *H. pylori* infection. However, we cannot rule out this possibility, as the predisposing conditions to *H. pylori* infection have not been clarified yet.

With regard to the potential aetio-pathogenetic role of *H. pylori* infection in COPD, the chronic activation of inflammatory mediators induced by *H. pylori* infection might lead to the development of COPD. The increased prevalence of CagA positive strains in our study population further supports this hypothesis. It is well known that these virulent strains stimulate the release of a variety of proinflammatory cytokines, including Interleukin-1 (IL-1), IL-8 and tumour necrosis factor-alpha.[26,27] Moreover, eradication of *H. pylori* leads to normalization of serum cytokines levels.[28] These cytokines are also thought to be involved in the pathogenesis of COPD.[29-31] Therefore, *H. pylori* infection in general and CagA-positive strains in particular might play a proinflammatory role and co-trigger COPD with other more specific environmental, genetic and unknown factors. The lack of association between spirometric values and *H. pylori* infection, reported in our study, suggest that *H. pylori* might have a minor role in the further progression of the disease.

Another potential pathogenetic mechanism could be the spilling or inhalation of *H. pylori* or its exotoxins into the respiratory tract, which also might lead to a chronic airway inflammation such as COPD. However, as far as we know, neither identification of *H. pylori* species in human bronchial tissue, nor isolation of *H. pylori* from bronchoalveolar lavage (BAL) fluid has been achieved yet.[32]

In conclusion, the present study suggests that patients with COPD have an increased seroprevalence of *H. pylori* infection. Our results must be confirmed in a larger number of patients. Further studies should be undertaken to clarify the pathogenetic mechanisms underlying the possible association between these diseases and the effect of *H. pylori* eradication on the natural history of COPD.

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Lactobacillus johnsonii La1 Attenuates *Helicobacter pylori*-Associated Gastritis and Reduces Levels of Proinflammatory Chemokines in C57BL/6 Mice

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In clinical settings, *Lactobacillus johnsonii* La1 administration has been reported to have a favorable effect on *Helicobacter pylori*-associated gastritis, although the mechanism remains unclear. We administered, continuously through the water supply, live La1 to *H. pylori*-infected C57BL/6 mice and followed colonization, the development of *H. pylori*-associated gastritis in the lamina propria, and the levels of proinflammatory chemokines *m*acrophage *i*nflammatory *p*rotein 2 (MIP-2) and *k*eratinocyte-derived *c*ytokine (KC) in the serum and gastric tissue over a period of 3 months. We documented a significant attenuation in both lymphocytic ($P = 0.038$) and neutrophilic ($P = 0.003$) inflammatory infiltration in the lamina propria as well as in the circulating levels of anti-*H. pylori* immunoglobulin G antibodies ($P = 0.003$), although we did not observe a suppressive effect of La1 on *H. pylori* colonizing numbers. Other lactobacilli, such as *L. amylovorus* DCE 471 and *L. acidophilus* IBB 801, did not attenuate *H. pylori*-associated gastritis to the same extent. MIP-2 serum levels were distinctly reduced during the early stages of *H. pylori* infection in the La1-treated animals, as were gastric mucosal levels of MIP-2 and KC. Finally, we also observed a significant reduction ($P = 0.046$) in *H. pylori*-induced interleukin-8 secretion by human adenocarcinoma AGS cells in vitro in the presence of neutralized (pH 6.8) La1 spent culture supernatants, without concomitant loss of *H. pylori* viability. These observations suggest that during the early infection stages, administration of La1 can attenuate *H. pylori*-induced gastritis in vivo, possibly by reducing proinflammatory chemotactic signals responsible for the recruitment of lymphocytes and neutrophils in the lamina propria.

Helicobacter pylori is a gram-negative spiral motile pathogen with a unique adaptive ability to colonize the hostile acidic environment of the stomach. Clinical manifestations of persistent chronic *H. pylori* infection range in severity from chronic active gastritis, chronic atrophic gastritis, and peptic ulceration to gastric mucosa-associated lymphoid tissue lymphoma and cancer. Early results from animal and clinical studies suggested that probiotics may contribute to the management of *H. pylori* infection (Maastricht 2-2000 Consensus) (35). Probiotics have been defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, Cordoba, Argentina, October 2001). Various *Lactobacillus* probiotic strains have been demonstrated to exert antagonistic activity against gram-negative pathogens (43) and *H. pylori* in particular. More specifically, administration of lactic acid- or butyric acid-producing bacteria, such as *Lactobacillus salivarius* (1, 31), *Lactobacillus gasseri* (50), and *Clostridium butyricum* (49), has been shown to prevent *H. pylori* colonization in germ-free mice. *Lactobacillus acidophilus* strain LB supernatants have been demonstrated to inhibit *Helicobacter felis* colonization in conventional specific-pathogen-free BALB/c mice (11). Furthermore, continuous administration of *Lactobacillus casei* strain Shirota over a 9-month period resulted in reduction of *H. pylori* colonizing numbers and concomitant attenuation of associated gastritis in C57BL/6 mice infected with *H. pylori* SS1 (Sydney strain 1) (44). In a similar study involving the same infection model, a mixture of *Lactobacillus rhamnosus* and *L. acidophilus* also inhibited *H. pylori* colonization and reduced the number of animals developing gastritis but did not affect *H. pylori*-induced apoptosis in the gastric mucosa (30).

Results from a number of clinical trials suggest that administration of lactic acid bacteria (LAB) can have a moderate, yet in some cases significant, inhibitory effect on *H. pylori* colonization as well as *H. pylori*-associated gastritis (for a review, see reference 27). In these studies, LAB have been administered to asymptomatic *H. pylori*-infected patients either alone (9, 14, 26, 37, 40) or as adjunctive agents to conventional eradication therapy (8, 13, 15, 21). However, the majority of studies utilized the urea breath test, a method which may not be suitable for assessment of *H. pylori* colonization in clinical settings involving lactobacilli, because of the ability of the lactobacilli to reduce *H. pylori* urease activity (1, 11, 44). Moreover, when more stringent methods, such as quantitative gastric cultures, were employed for evaluation of *H. pylori* colonization, no significant effect was observed (40). Despite the conflicting reports of the ability of lactobacilli to affect *H. pylori* colonization, one interesting common observation was the significant attenuation of *H. pylori*-associated inflammation which was

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evident with the lactobacillus-administered groups. In particular, *Lactobacillus johnsonii* La1 (Nestlé, Switzerland) has been shown to exert such an anti-inflammatory effect in two double-blind, placebo-controlled clinical trials, where an acidified milk containing live La1 cells (LC-1) was administered alone (40) or as adjunct to antibiotic eradication therapy (21) to *H. pylori*-positive asymptomatic volunteers.

Specific CXC family chemokines, such as interleukin-8 (IL-8), growth-related oncogene alpha (GRO-α), gamma interferon-inducible protein 10 (IP-10), and monokine induced by gamma interferon (MIG), have been associated with the development of *H. pylori* gastritis during early infection stages (18, 54) by driving the chemotaxis of inflammatory cells such as neutrophils (IL-8 and GRO-α) and T lymphocytes (IP-10 and MIG). In rodents, murine macrophage inflammatory protein 2 (MIP-2) and Gro-α/keratinocyte-derived cytokine (KC) have been shown to possess distinct sequence homology to human GRO-α, -β, and -γ chemokines and are regarded as the murine functional homologs of human IL-8 (17, 42).

In the present study, we explored the potential anti-inflammatory effect of La1 on *H. pylori*-associated gastritis by the *H. pylori* SS1 strain infection model with C57BL/6 mice, following La1 continuous administration over a period of 3 months. We observed, following histological evaluation, a significant reduction in *H. pylori*-associated gastritis and reduced levels of proinflammatory chemokines MIP-2 and KC in the serum and in gastric explants from the animals. Similarly, lactobacillus spent culture supernatants significantly reduced *H. pylori*-induced secretion of IL-8 by human gastric epithelial cells in vitro.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Lactobacillus johnsonii* La1, *Lactobacillus amylovorus* DCE 471, and *Lactobacillus acidophilus* IBB 801 were part of the lactic acid bacteria of the PROPATH collection, kindly provided by Luc De Vuyst (Free University, Brussels). They were grown in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, Mich.) overnight (o/n) at 37°C (approximately 10^{09} CFU ml^{-1}) prior to use. Respective cell-free spent culture supernatants (LAB-SCS) were prepared by centrifugation of liquid LAB cultures at 10,000 × *g* for 30 min at 4°C, filtered through a 0.22-μm-pore-size filter (Millipore, Molsheim, France), and checked for the absence of bacteria by plating on MRS agar. They were kept frozen at −20°C until use.

Early-passage *H. pylori* strain SS1 was kindly provided by R. L. Ferrero (Institut Pasteur, Paris, France). *Helicobacter pylori* strains CCUG 38770 (Culture Collection, University of Göteborg, Sweden) and 009A were generously provided by T. Wadström (University of Lund, Sweden) and A. L. Servin (INSERM U510, Châtenay-Malabry, France), respectively. All *H. pylori* strains were routinely cultured under microaerophilic conditions (CampyPak-Plus; Becton-Dickinson, Cockeysville, Md.) at 37°C on Wilkins-Chalgren agar enriched with 7% (vol/vol) horse blood and 1% (vol/vol) VITOX (Oxoid, Basingstoke, United Kingdom). Unless otherwise stated, highly motile bacillary *H. pylori* cells derived from fresh o/n cultures were used for all in vitro and in vivo procedures.

***H. pylori* infection of C57BL/6 mice and LAB administration.** Specific-pathogen-free 6- to 8-week-old male 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 (33, 44). Briefly, freshly prepared aliquots (100 μl, 10^{8} CFU) of *H. pylori* SS1 strain in brain heart infusion broth (Oxoid) were administered to mice via orogastric inoculation three times within a week (days 1, 3, and 5). Accordingly, all noninfected control animals were inoculated with the same volume of plain brain heart infusion broth. LAB cultures were administered through the animals' drinking water, starting from the day of the last *H. pylori* challenge (day 5), over a period of 3 months. Daily water consumption and LAB viability in the water were monitored closely. Differences in the volumes consumed, possibly due to taste variations or potential osmolality changes following the addition of lactobacilli, between the animal groups were recorded and taken into account for the determination of the administered daily dose. The following groups of animals were included in the study: *H. pylori*-infected mice administered *L. johnsonii* La1 (Hp-La1 group; $n = 15$), *L. amylovorus* DCE 471 (Hp-DCE471 group; $n = 15$), or *L. acidophilus* IBB 801 (Hp-IBB801 group; $n = 15$). The control groups were *H. pylori*-infected mice left untreated (HpSS1 group; $n = 15$) or given nonfermented MRS medium (Hp-MRS group; $n = 15$). We also included uninfected mice ($n = 15$), as well as mice administered only the lactobacilli, namely, *L. johnsonii* La1 (La1 group; $n = 15$), *L. amylovorus* DCE 471 (DCE 471 group; $n = 15$), and *L. acidophilus* IBB 801 (IBB 801 group; $n = 15$). Mean daily lactobacillus consumption per animal was calculated to be 1.5×10^{8} CFU for La1-administered groups, 2.1×10^{8} CFU for DCE 471-administered groups, and 4.6×10^{8} CFU for IBB 801-administered groups. At time intervals of 1, 6, and 12 weeks, blood samples were collected and five animals per group were sacrificed by cervical dislocation. All methods describing assessment of *H. pylori* colonization, LAB isolation, and identification in gastric and intestinal samples as well as evaluation of gastritis and anti-*H. pylori* immunoglobulin G (IgG) response have been described in detail before (44). Levels of MIP-2 and KC in mouse serum were determined by enzyme-linked immunosorbent assay (ELISA) Quantikine immunoassay kits (R&D Systems, Minneapolis, Minn.), according to the manufacturer's protocols. Analysis of the results with respect to *H. pylori*-associated gastritis was performed by the Wilcoxon rank-sum test. Serum MIP-2 and KC levels were compared between study and control groups by two-tailed unpaired *t* test with Welch correction.

Determination of proinflammatory chemokines in gastric organ cultures from *H. pylori*-infected animals administered La1. MIP-2 and KC secreted by the gastric mucosa were measured following a modification of the protocol of Stingmund et al. (46). Uninfected mice or mice infected with *H. pylori* were administered La1 as described in the previous section. At selected time points (days 6, 8, 12, and 18) following the first *H. pylori* challenge, blood samples were collected and eight animals per group were sacrificed by cervical dislocation. Excised stomachs were dissected and turned inside out. They were then washed in cold phosphate-buffered saline supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate (Sigma-Aldrich, Steinheim, Germany) and were placed in 24-well flat-bottomed culture plates (Greiner Bio-One, Frickenhausen, Germany) in 2 ml serum-free RPMI 1640 medium (Gibco, Inc., Grand Island, N.Y.) supplemented with penicillin and streptomycin. The culture medium was collected after 24 h of incubation at 37°C, centrifuged at 15,000 × g, and stored at −20°C until analyzed by ELISA (Quantikine immunoassay) for the presence of MIP-2 and KC. Analysis of the results was carried out by two-tailed unpaired *t* test with Welch correction.

In vitro *H. pylori* infection of AGS cells and determination of IL-8 levels. Human gastric adenocarcinoma cell line AGS (ATCC CRL 1739) was maintained in F-12 Coon's modification medium (Earnsclone, Ltd., United Kingdom) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37°C in a 5% CO_2 atmosphere. For the infection studies, AGS cells were seeded at a density of 1.7×10^{5} cells per well and incubated o/n. Prior to infection with *H. pylori*, they were washed twice with fresh cell culture medium without antibiotics, and the media were replaced with antibiotic-free complete F-12 (10% fetal bovine serum). *H. pylori* bacteria were harvested from 18-hour solid cultures and resuspended in antibiotic-free complete F-12 medium. We standardized the *H. pylori* to AGS populations to achieve a multiplicity of infection of 100. In order to assess the effect of lactobacilli on *H. pylori*-induced IL-8 secretion, we simultaneously incubated live lactobacilli with *H. pylori*-infected AGS cells. However, we observed a massive destruction of the epithelial cell monolayer following a 24-hour incubation (data not shown). We therefore incubated *H. pylori* with LAB-SCS at a 1:1 (vol/vol) ratio for 1 h at 37°C under microaerophilic conditions, which allow for optimum *H. pylori* viability. Subsequently, the whole bacterial suspension was added to AGS monolayers and further incubated for 24 h (37°C in a 5% CO_2 atmosphere). AGS supernatants were collected, centrifuged at 15,000 × *g*, and kept at −20°C until assayed for IL-8. LAB-SCS were used at their native pH of 4.5, as well as neutralized to pH 6.8 by addition of NaOH. Appropriate controls of acidified nonfermented MRS adjusted to pH 4.5 with 1 N hydrochloric acid or DL-lactic acid (final concentration, 100 mM; Sigma-Aldrich) were also included in the study. The corresponding controls of LAB-SCS at neutralized pH 6.8 were nonfermented MRS broth and MRS initially acidified with lactic acid to pH 4.5 and readjusted to pH 6.8 with 1 N NaOH. In all experiments, we assessed *H. pylori* morphology by microscopic observation and viability by plating serial dilutions of bacterial cultures on Wilkins-Chalgren agar. IL-8 levels in the AGS culture supernatants were determined by ELISA (Bender MedSystems, Vienna, Austria) according to the manufacturer's proto-

TABLE 1. Chronic inflammatory infiltration[a] in *H. pylori*-infected mice treated with lactobacilli

Wk	Group	No. of mice of grade[b]:				*P*[c]
		0	1	2	3	
6	HpSS1	0	5	0	0	
	Hp-DCE471	3	2	0	0	*0.047*
	Hp-La1	5	0	0	0	*0.003*
	Hp-IBB801	4	1	0	0	*0.014*
12	HpSS1	0	3	2	0	
	Hp-DCE471	0	4	1	0	0.264
	Hp-La1	2	3	0	0	*0.038*
	Hp-IBB801	0	4	1	0	0.264

[a] Lymphocyte infiltration in the antrum.
[b] Histopathology grades according to the updated Sydney system (16) are as follows: normal, 0; mild, 1; moderate, 2; and marked, 3.
[c] Statistical analysis with reference to HpSS1 control group, done by Wilcoxon rank sum test. Significant correlations are depicted in italics.

TABLE 2. Activity of chronic gastritis[a] in *H. pylori*-infected mice treated with lactobacilli

Wk	Group	No. of mice of grade[b]:				*P*[c]
		0	1	2	3	
6	HpSS1	0	5	0	0	
	Hp-DCE471	3	2	0	0	*0.047*
	Hp-La1	5	0	0	0	*0.003*
	Hp-IBB801	4	1	0	0	*0.014*
12	HpSS1	0	0	4	1	
	Hp-DCE471	0	4	1	0	*0.011*
	Hp-La1	4	1	0	0	*0.003*
	Hp-IBB801	0	4	1	0	*0.011*

[a] Neutrophil infiltration in the antrum.
[b] Histopathology grades according to the updated Sydney system (16) are as follows: normal, 0; mild, 1; moderate, 2; and marked, 3.
[c] Statistical analysis with reference to HpSS1 control group, done by Wilcoxon rank sum test. Significant correlations are depicted in italics.

col. Absorbance at 450 nm (corrected for background at 620 nm) was measured with a Sunrise microtiter plate reader (Tekan, Grödig, Austria). Results were analyzed by two-tailed unpaired *t* test.

RESULTS

Effect of lactobacilli on *H. pylori*-infected and noninfected animals. Animals showed no signs of discomfort or distress throughout the whole observation period. No differences in weight between animal groups were recorded over the observation period (data not shown), an indication that there was no adverse effect to the animals by the administration of lactic acid bacteria at such high concentrations.

We followed qualitatively the kinetics of the administered lactobacilli, isolating them from the wet feces of the animals. We identified several other commensal lactobacilli, such as *Lactobacillus reuteri*, *L. gasseri*, and *Lactobacillus jensenii*, in the murine gastric and intestinal microflora by sugar fermentation profiling (AP 150 CHL) and 16S to 23S rRNA typing (data not shown).

Evaluation of chronic and chronic active gastritis in *H. pylori*-infected animals. Histopathologic evaluation of the gastric mucosa at 6 weeks postinfection revealed an induction of mild chronic gastritis (Tables 1 and 2) in the antra of animals in the HpSS1 control group. All gastric samples were characterized by mild infiltration of the lamina propria with scattered lymphocytes and neutrophils. At 12 weeks, we observed that two animals developed moderate chronic gastritis (Table 1) without formation of lymphoid follicles. However, there was an increase in active inflammation levels, as four animals developed moderate and one animal marked chronic active gastritis (Table 2). Development of glandular atrophy and intestinal metaplasia was not observed, as the time interval from the onset of infection was too short. No significant difference in levels of *H. pylori*-associated gastritis between the HpSS1 and Hp-MRS animal groups was observed (data not shown). Uninfected control animals developed no evidence of chronic or chronic active gastritis. A significant attenuation in chronic gastritis at 6 weeks was evident for all lactobacillus-treated *H. pylori*-infected animal groups, compared to the HpSS1 control (Table 1). This was more pronounced with the Hp-La1 group, where none of the animals developed changes of chronic gastritis ($P = 0.003$), followed by Hp-IBB801 (one animal with mild gastritis; $P = 0.014$) and Hp-DCE471 (two animals with mild gastritis; $P = 0.048$). However, at 12 weeks only the Hp-La1 mice maintained significantly mild changes of chronic gastritis (three animals with mild chronic gastritis; $P = 0.038$) compared to HpSS1 (Table 1). A trend towards reduced inflammatory lymphocytic infiltration of the gastric mucosa was also observed with the Hp-DCE471 and Hp-IBB801 groups, albeit never reaching statistical significance.

A pronounced reduction in the neutrophilic polymorphonuclear infiltration of the lamina propria was observed to occur in the antra of all lactobacillus-administered *H. pylori*-infected animals at 6 and 12 weeks (Table 2). Mice belonging to the Hp-La1 group showed no evidence of active inflammation at 6 weeks, and only one mouse developed mild changes at 12 weeks ($P = 0.003$). Two animals in the Hp-DCE471 group and one animal in the Hp-IBB801 group developed mild changes of active gastric inflammation at 6 weeks. Moreover, all but one animal in both groups developed mild active inflammatory infiltration at 12 weeks compared to the moderate or marked active inflammation observed for the HpSS1 control group (Table 2). No signs of chronic active inflammatory infiltration were observed for the control animal groups administered just the lactobacilli (data not shown). Therefore, continuous administration of lactobacilli during the early stages of *H. pylori* infection can affect monocytic (La1) as well as neutrophilic (La1, DCE 471, and IBB 801) polymorphonuclear infiltration in the lamina propria and thus may contribute to the attenuation and delayed onset of *H. pylori*-associated gastritis.

Effect of lactobacilli on the humoral anti-*H. pylori* response. *H. pylori*-specific IgG antibodies are the dominant antibody class present in the sera of chronically infected mice (22) and may serve as an indicator of successful *H. pylori* infection. We measured anti-*H. pylori* IgG antibody titers in serum and observed a trend towards reduced titers in all lactobacillus-treated groups. More specifically, for the Hp-La1 group, we documented a significant reduction in anti-*H. pylori* titers at 12 weeks ($P = 0.003$) (Fig. 1A). Interestingly, for the Hp-IBB801 group, titers were significantly reduced at 6 weeks ($P = 0.027$) but not at 12 weeks (Fig. 1B). Finally, in the Hp-DCE471

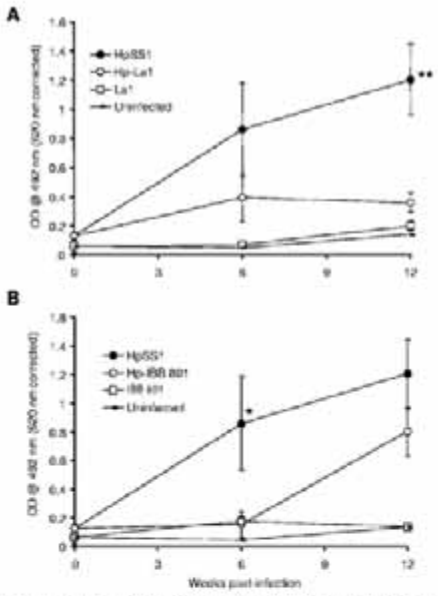

FIG. 1. Anti-*H. pylori* IgG antibody responses in *H. pylori*-infected animal groups Hp-La1 (A) or Hp-IBB801 (B) and HpSS1. Uninfected control mice having received La1 (A) or IBB 801 (B) are also depicted. Mouse sera were diluted to 1:50. A statistically significant decrease in anti-*H. pylori* titers was observed at 12 weeks postinfection for Hp-La1 mice (A) and at 6 weeks for Hp-IBB801 mice (B). A nonsignificant trend towards decreased anti-*H. pylori* titers was observed for IBB 801 at 12 weeks postinfection. OD, optical density; *, P value of <0.05; **, P value of <0.01.

group, there was a trend towards reduced anti-*H. pylori* titers throughout the whole experimental period, but the results never reached significance (data not shown).

Effect of lactobacilli on *H. pylori* colonization. We detected a significant increase in the numbers of *H. pylori* colonizing bacteria in the HpSS1 control group throughout the 12-week observation period (Fig. 2). In the initial stages of infection (day 6), populations of *H. pylori* detected in the gastric tissue were in the range of 10^4 CFU g^{-1}, rising to about 10^7 CFU g^{-1} by week 6 and 10^8 CFU g^{-1} by week 12. These results were consistent with the histopathologic observations, where *H. pylori* colonization was evaluated as much heavier (moderate or marked) at 6 and 12 weeks postinfection than at the beginning of the experiment (none or mild). No reduction in *H. pylori* colonizing levels throughout the whole observation period was determined for the lactobacillus-treated *H. pylori*-infected animal groups, assessed by either determination of quantitative cultures (Fig. 2) or histological evaluation (data not shown). Furthermore, an equal trend towards an increase in *H. pylori* colonizing numbers was observed for all animal groups, suggesting that the administered lactobacilli did not suppress *H. pylori* SS1 strain colonization in vivo.

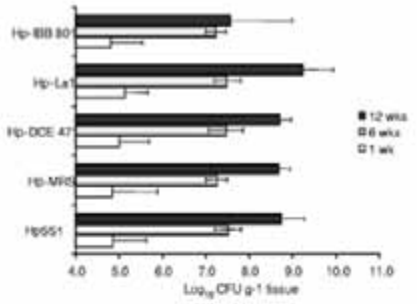

FIG. 2. *H. pylori* colonization in *H. pylori*-infected mice, following continuous administration of lactobacilli. Each time point (1 week [wk], 6 weeks [wks], and 12 wks) and bar represents the average of five animals. No significant reduction in *H. pylori* colonization due to administration of lactobacilli was observed for any group at any time point compared to the untreated *H. pylori*-infected study group.

Effect of lactobacilli on proinflammatory chemokine levels in serum and gastric mucosa. MIP-2 and KC are the dominant IL-8-like chemokines in inflammatory states in rodents and potent murine neutrophil chemoattractants (2, 6). We determined MIP-2 and KC levels in the serum samples from the animals and found significantly reduced levels of MIP-2 (Fig. 3A) but not KC (data not shown) at 6 weeks. More specifically, MIP-2 was high in serum during initial *H. pylori* infection stages (day 6) before the administration of lactobacilli and remained increased at 6 weeks postinfection for the HpSS1 control animals ($P = 0.0412$) but not for the Hp-La1 group (Fig. 3A). There was a similar trend towards reduced MIP-2 levels at 6 weeks with the Hp-DCE471 and Hp-IBB801 groups, without reaching significance (data not shown). We did not observe any increase in MIP-2 or KC levels with the lactobacillus-administered uninfected animal groups or the control uninfected mice.

We further assessed the levels of MIP-2 and KC in the gastric mucosae of *H. pylori*-infected animals during the first 3 weeks of *H. pylori* infection. We observed a time-dependent increase in the levels of MIP-2 starting at day 8 (Fig. 3B), whereas KC was detected as early as day 6 postinfection (Fig. 3C) in all *H. pylori*-infected animals. Both chemokines seemed to reach a plateau by day 19 postinfection. However, with the La1-treated *H. pylori*-infected animals, we observed a distinct delay in induction, reflected by significantly lower levels of both MIP-2 (day 8, $P = 0.025$; day 12, $P = 0.040$) and KC (day 6, $P = 0.006$; day 8, $P = 0.008$) (Fig. 3B and C, respectively). To ascertain that this observation was not due to reduced *H. pylori* colonization, we determined *H. pylori* viability in the gastric samples and found no differences between the groups with or without La1 administration (data not shown).

Effect of lactobacilli on *H. pylori*-induced IL-8 secretion by gastric epithelial cells. Upon infection of AGS cells with *H. pylori* strains CCUG 38770 and 069A, significantly higher levels of IL-8 were induced at 24 h postinfection (636.5 pg ml^{-1}, $P < 0.0001$, and 605.6 pg ml^{-1}, $P < 0.0001$, respectively) than for uninfected AGS cells (11.8 pg ml^{-1}). However, *H. pylori* strain SS1 induced 12-fold-lower IL-8 levels, which did not differ

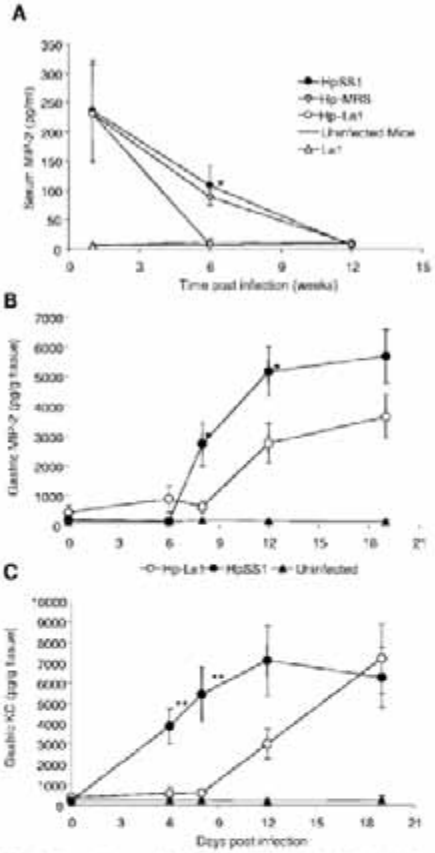

FIG. 3. Levels of proinflammatory cytokines in *H. pylori*-infected mice, following continuous administration of La1. (A) Serum MIP-2 protein levels in HpSS1, Hp-MRS, and Hp-La1 animal groups. Results for uninfected control mice and mice administered La1 are also depicted. Each point is the average of five animals. (B and C) Gastric MIP-2 (B) and KC (C) levels in *H. pylori*-infected mice with or without treatment with La1. Each point is the average of eight animals. Statistical analysis was carried out with respect to the untreated *H. pylori* controls (two-tailed unpaired *t* test, Welch correction).

from basal levels produced by uninfected AGS cells. Bacillary and highly motile bacteria yielded higher IL-8 levels, whereas the presence of coccoid forms reduced the levels of induction (data not shown).

Following treatment with LAB-SCS at their native pH of 4.5, *H. pylori* strains were significantly affected in their ability to induce IL-8 by AGS cells, with concomitant reduction of their viability. More specifically, La1 supernatant (pH 4.5) reduced viability of *H. pylori* strains CCUG 38770 and 069A by nearly 5 $\log_{10}$ CFU ml^{-1} (Table 3) and significantly depressed their ability to induce IL-8 ($P = 0.006$ and $P = 0.029$, respectively) (Fig. 4A and B, respectively). A weaker though significant loss in viability (2 to 3 $\log_{10}$ CFU ml^{-1} [Table 3]) and reduction in IL-8 levels ($P = 0.021$) were observed when *H. pylori* CCUG 38770 was treated with DCE 471 (Fig. 4A). In order to exclude potential pH-dependent activity on *H. pylori* viability, we repeated the experiments with neutralized (pH 6.8) LAB-SCS and observed a significant reduction of IL-8 secretion in the case of *H. pylori* CCUG 38770 treated with La1 supernatant ($P = 0.046$) (Fig. 4C). A trend towards reduced IL-8 levels was also observed following treatment of *H. pylori* 069A with La1 supernatant, yet without reaching significance (Fig. 4D). In all experiments involving treatment with neutralized LAB-SCS, we did not observe any loss of *H. pylori* viability (Table 3), changes in its morphology and motility, or reduction in its ability to adhere to the gastric epithelial cells (B. Martinez-Gonzalez, unpublished data).

DISCUSSION

In the present study, we utilized an established animal model of *H. pylori* infection to evaluate the effect of continuous lactobacillus administration on *H. pylori* colonization and development of associated chronic gastritis. This model involves the mouse adapted *H. pylori* strain SS1, which colonizes the C57BL/6 mouse heavily and leads to the development of appreciable levels of gastritis closely mimicking human *H. pylori* infection (22, 32, 33, 48). We have applied this infection model in the past and have studied the effect of *L. casei* Shirota on *H. pylori* infection over a period of 9 months (44). In the present study, we have limited our observations to a period of 3 months because chronic active gastritis is more pronounced in this particular mouse strain during this period (34, 44).

Continuous administration of lactobacilli La1, DCE 471, and IBB 801 did not reduce the *H. pylori* colonizing numbers over this experimental period. In fact, bacterial numbers were increased during the course of the study, an observation made independently by determination of viable bacterial counts and

TABLE 3. Viability of *H. pylori* strains CCUG 38770 and 069A following 1-h treatment with LAB-SCS at their native pH of 4.5 or neutralized to pH 6.8

pH	Treatment[a]	Viability ($\log_{10}$ CFU ml^{-1} ± SEM[b] of *H. pylori* strain	
		CCUG 38770	069A
4.5	Untreated	8.1 ± 0.11	7.7 ± 0.11
	La1	3.3 ± 0.52**	<2**
	DCE 471	7.5 ± 0.13*	5.4 ± 0.23**
	IBB 801	7.8 ± 0.06	6.3 ± 0.17*
	MRS-LA	7.9 ± 0.09	6.9 ± 0.27
6.8	Untreated	8.1 ± 0.07	7.8 ± 0.20
	La1	7.9 ± 0.09	7.1 ± 0.27
	DCE 471	7.8 ± 0.20	7.3 ± 0.19
	IBB 801	7.7 ± 0.31	7.3 ± 0.14
	MRS-LAn	7.5 ± 0.53	7.4 ± 0.17
	MRS	7.9 ± 0.02	7.3 ± 0.26

[b] *, P value of <0.05; **, P value of <0.01 (compared to the respective untreated sample [unpaired *t* test with Welch correction]).

[a] MRS-LA, acidified non-fermented MRS adjusted to pH 4.5 with DL-lactic acid. MRS-LAn, MRS initially acidified with lactic acid to pH 4.5 and readjusted to pH 6.8 with 1 N NaOH.

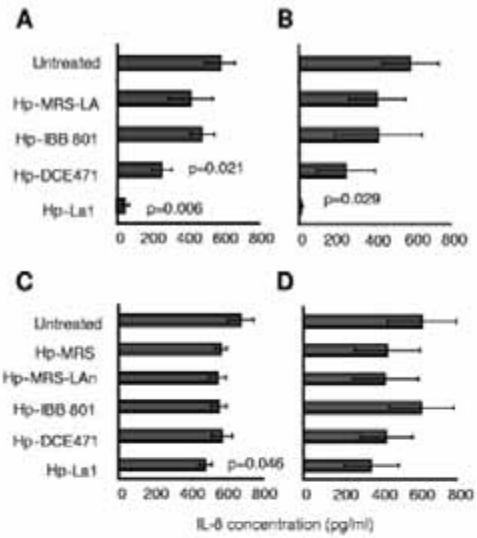

FIG. 4. *H. pylori*-induced IL-8 secretion by AGS cells, following treatment with LAB-SCS, at their native pH of 4.5 (A and B) or at a neutralized pH of 6.8 (C and D). *H. pylori* strains depicted are CCUG 38770 (A and C) and 069A (B and D). Statistical analysis was carried out with respect to the untreated *H. pylori* controls (two-tailed unpaired *t* test).

histopathologic assessment of density of colonization. There are conflicting reports regarding the antimicrobial properties of lactobacilli. *L. johnsonii* La1, in particular, has been reported to exert antibacterial activity in vivo in conventional and germ-free mice infected with *Salmonella enterica* serovar Typhimurium as well as in vitro against a wide range of gram-negative and gram-positive pathogens, but not *H. pylori* (5). This antibacterial activity was attributed to a yet-unidentified nonbacteriocin substance which acted independently of lactic acid production. Clinical studies involving administration of La1 to asymptomatic *H. pylori*-positive volunteers, either alone (14, 26) or as adjunct agent to eradication therapy (21, 37), suggested a marginal effect on *H. pylori* colonization. However, in these studies, evaluation of *H. pylori* colonization was carried out by [^{13}C]urea breath test and not by direct quantitative cultures, which would have presented a far more accurate result. In a double-blind, placebo-controlled randomized clinical trial in which colonization was evaluated by quantitative bacterial cultures, no difference in *H. pylori* colonization levels between the placebo- and the La1-administered volunteers was observed (40). Our data from the SS1 mouse model with reference to *H. pylori* colonization are in accordance with the latter findings and suggest that La1 may not exert an antimicrobial activity on *H. pylori* in vivo, despite reports about production of substances of a bacteriocin (3) or nonbacteriocin (5) nature. Furthermore, it would seem highly unlikely that an actively secreted bacteriocin produced by La1 would retain activity, with the abundance of proteolytic activity present in the gastric epithelium. Regarding DCE 471 and IBB 801, there have been no previous reports of in vitro or in vivo anti-*H. pylori* activity. DCE 471 initially isolated from corn steep liquor has been shown to produce the bacteriocin amylovorin 471, with a narrow antibacterial spectrum against gram-positive bacteria (7) and a very strong similarity within its 35-amino-acid N-terminal sequence with lactacin X, a product of *L. johnsonii* VP 11088 (23). Similarly, IBB 801 has been shown to produce a 6.5-kDa bacteriocin, named acidophilin 801, which displays a narrow inhibitory activity towards related lactobacilli but not gram-negative pathogenic bacteria (55).

We evaluated anti-*H. pylori* IgG titers in our mice primarily as a sign of successful *H. pylori* infection, as IgG is the dominant antibody class present in the sera of chronically infected mice (22). IgG levels in the La1-treated animals were significantly lower than levels in untreated, *H. pylori*-infected controls. For humans, significant decrease in IgG antibody titers has been suggested as an indicator for successful eradication of *H. pylori* (4, 20). In the past, we observed *L. casei* strain Shirota-mediated decrease in anti-*H. pylori* titers in C57BL/6 mice infected with the SS1 strain, but we attributed that effect to the inhibition of *H. pylori* colonization (44). Others have also documented a reduction in IgG antibodies following administration of lactobacilli to *H. pylori*-infected mice, as a result of decreased *H. pylori* colonization (1). However, in the present study, administration of La1 and to a lesser extent IBB 801 or DCE 471 significantly reduced IgG titers without concomitant decrease in *H. pylori* colonizing numbers. This may suggest that administration of lactobacilli and La1 in particular could potentially affect the humoral immune response towards *H. pylori* through possible induction of TH2 cellular subsets participating primarily in humoral responses. However, we did not evaluate IgG1/IgG2a subtype ratios in the sera of our mice because commercially available IgG2a sera, used for the isotype determination raised in BALB/c mice, fail to detect or grossly underestimate levels of IgG2c present in C57BL/6 mice (36).

In our experiments, although we did not observe any anti-*H. pylori* activity, a distinct attenuation in the neutrophilic polymorphonuclear inflammatory infiltration of the laminae propriae of the lactobacillus-administered animals was evident. Neutrophilic infiltration in the lamina propria is regarded as a hallmark of *H. pylori*-associated gastritis, and the density of intraepithelial neutrophilic infiltration has been well linked to the extent of mucosal damage and the intensity of *H. pylori* infection (47). Invading neutrophils can be a very sensitive indicator of *H. pylori* presence and disappear within days of cure of the infection. Furthermore, in the case of La1-treated animals there was an equally significant reduction in the levels of intramucosal infiltrating lymphocytes. This is an important observation, as chronic inflammatory cells have been shown to disappear slowly following *H. pylori* eradication and usually persist for a long time before they fall to the expected or "normal" levels in humans (25). These results suggest that lactobacilli and La1 in particular may exert an anti-inflammatory activity without necessarily affecting *H. pylori* colonization. Similar anti-inflammatory properties with concomitant reduction in proinflammatory cytokine expression, without a profound effect on pathogen colonization, have been observed with a murine *Helicobacter hepaticus*-induced inflammatory

bowel disease model following administration of *Lactobacillus* spp. (41).

In humans, the recruitment and activation of inflammatory cells in the *H. pylori*-infected gastric mucosa is mediated by proinflammatory CXC chemokines, such as IL-8, GRO-α, IP-10, and MIG (12, 18, 45). Moreover, successful *H. pylori* eradication has been shown to result in significant reduction of mucosal IL-8 and GRO-α to nondetectable levels (54). Although mice lack a sequence homolog of human IL-8, chemokines such as MIP-2 and KC show distinct sequence homology to human GRO-α, -β, and -γ chemokines and are regarded as the murine functional homologs of human IL-8 (17, 42). Both have been reported to be very potent neutrophil attractants and activators, thus functioning as the major proinflammatory CXC chemokines in mice (2, 6, 19). A putative murine homolog of the human IL-8 receptor β, to which both mouse MIP-2 and KC bind with high affinity (28), has also been cloned.

Gastric transcriptional induction of these chemokines at the early stages of *H. pylori* infection in vitro and in vivo has been documented (18, 24, 38, 39), but there is no information describing levels of gastric and systemic MIP-2 and KC chemokines during early infection in the *H. pylori* SS1 model. We determined significantly higher levels of MIP-2 protein in the sera of the *H. pylori*-infected controls than in the sera of the lactobacillus-treated animals during the early stages of infection, namely, week 1 and week 6 postinfection. However, there was no difference in the levels of MIP-2 in serum at week 12 postinfection, by which time the *H. pylori*-infected control animals had already developed marked chronic active gastritis. Furthermore, in separate experiments we followed the kinetics of MIP-2 and KC protein secretion in murine gastric explants during the initial 3 weeks of *H. pylori* infection. Our findings indicate potential differences in the temporal expression of these two chemokines in the gastric mucosa, as the expression of KC preceded that of MIP-2 in the first days following *H. pylori* infection. A similar temporal pattern of KC and MIP-2 expression has been documented for inflammatory conditions induced by surgical injury in C57BL/6 mice (19), as a consequence of temporally ordered contribution of nonmyeloid and myeloid cell types involved in the expression of these two chemokines (2). Relevant spatial differences in the expression levels of GRO-α and IL-8 chemokines have been demonstrated for *H. pylori*-infected individuals (18). With the La1-treated animals, we observed a significant delay in the production of both MIP-2 and KC, as gastric mucosal levels of both chemokines were very low during the first 10 days postinfection. This could suggest a potential effect of La1 on temporal and/or spatial expression of these two chemokines by targeting specific cellular types. However, from this study we cannot draw any such conclusions, since we did not characterize the specific cellular populations expressing MIP-2 and KC in our mice.

Consistent with our in vivo observations that La1 administration reduced gastric mucosal levels of MIP-2 and KC, we also observed a significant decrease in IL-8 levels secreted by human gastric epithelial cells infected with *H. pylori* in vitro. IL-8 is the chief mediator of inflammatory responses during *H. pylori* infection in humans, the mucosal levels of which have been correlated with cellular inflammatory infiltration in the antrum (54). We observed that cell-free spent culture supernatants of La1 and DCE 471 at their native pH of 4.5 could dramatically reduce levels of IL-8 released in vitro by *H. pylori*-infected AGS cells. However, this was not a direct effect on IL-8 secretion, as La1 and in part DCE 471 induced stress-related morphological changes (U-shaped and coccoid formations) and significantly reduced *H. pylori* viability. When we repeated the experiments using supernatants neutralized to pH 6.8, we observed that only La1 still caused a significant reduction in IL-8 secretion by gastric epithelial cells. As we did not observe any reduction in *H. pylori* viability or changes in bacterial morphology and the binding efficiency of *H. pylori* to AGS cells (B. Martinez-Gonzalez, unpublished data), this could suggest that an La1-secreted compound(s) could potentially interfere with IL-8 induction directly. Indeed, previous reports indicate that La1-derived lipoteichoic acid or peptidoglycan trace contaminants in the lipoteichoic acid preparations can antagonize, in a dose-dependent manner, IL-8 production by human intestinal epithelial HT-29 cells in response to lipopolysaccharide and gram-negative bacteria (52). In *H. pylori* infection, NF-κB-dependent IL-8 induction is mediated by intracellular Nod1 through recognition of *meso*-diaminopimelate-containing tripeptidoglycan, transferred inside the epithelial cells through the type IV secretion system (51). Nod proteins have been proposed to function as intracellular sensors by which epithelial cells can discriminate pathogenic from nonpathogenic bacteria (10, 29). Consequently, an La1-secreted compound(s) may interfere with *H. pylori* peptidoglycan recognition by Nod1 or direct activation of Nod2 signaling. This could lead to attenuation of *H. pylori*-associated gastritis through possible downregulation of TH1 responses (53).

We observed a pronounced anti-inflammatory effect exerted by lactobacilli and La1 in particular on *H. pylori*-associated neutrophilic and lymphocytic infiltration and were able to document significantly reduced proinflammatory chemokine levels in the gastric mucosa during the early stages of *H. pylori* infection. It would be of great interest to further explore the role of such probiotic strains in the complex regulation of proinflammatory signal strength during early infection and identify the clinical potential in the induction of cellular inflammatory processes.

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In vitro inhibition of *Helicobacter pylori* by micromycetes

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Abstract

The anti-*Helicobacter pylori* effect of 22 micromycetes were studied against one standard strain and 11 clinical isolates of *H. pylori*. *Penicillium ochlochloron* and *Penicillium funiculosum* have been proven the most active fungi against this microorganism. Further bio-guided chemical analysis of *P. funiculosum* afforded an active component identified as (−) 2,3,4-trihydroxybutanamide.

Keywords: Anti-*Helicobacter pylori* activity; Micromycetes; (−) 2,3,4-trihydroxy-butanamide

1. Introduction

Among the pathogenic bacterial species of the gastrointestinal system, *Helicobacter pylori* is a relevant agent of chronic gastritis, peptic ulceration and gastric cancer in humans [1–5]. The gram-negative curved rod bacterium colonizes the gastric epithelial surface, survives and multiplies in the stomach due to its specific enzyme mechanisms and causes acute and chronic inflammatory response to the host [6]. In addition, it produces numerous virulence factors, the most important being urease, catalase, VacA and cagA.

It is known that *H. pylori* could be eradicated by mixing therapeutic agents such as antibiotics, bismuth subsalicylate, proton pump inhibitors and H_2-blockers [7]. Approximately, 15–30% of infected patients experience therapeutic failure with multi-drug therapy internationally. Increasing resistance to commonly used antibiotics seems to be the main reason for this [8]. In view of the incomplete achieved cure and possible side effects, we decided to look for further chemotherapeutic agents able to inhibit the growth of *H. pylori* in vitro. The concept that substances derived from one living organism may affect another organism is old. Some of the secondary metabolites that occur in fungi are fairly widespread, but many are confined to a few species. Hence, screening of further fungi species usually leads to the discovery of new bioactive secondary metabolites. The broad diversity of the fungi, as well as its easy acquisition makes them especially interesting for natural products screening program.

Particularly desirable is the discovery of novel prototype antimicrobial agents representing new chemical classes that operate by different modes of action than

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existing antibacterial agents and, consequently, lack cross-resistance to chemicals currently used.

2. Material and methods

2.1. Fungi

The fungal species used for the present investigation are seen in Table 1.

The organisms have been obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological research "Sinisa Stankovic" (IBISS), Belgrade, Yugoslavia and the Centre for Preventive Medicine, VMA.

The micromycetes were maintained on malt (1 l H_2O + 50 g yeast extract) and Potato–Dextrose (1 l H_2O + 200 g potato + 15 g dextrose) liquid medium using Erhlenmeyer flasks at 25 °C for 21 days, while dermatomycetes were grown on Sabouraud (1 l H_2O + glucose 40 g + peptone 10 g) liquid medium at 25 °C for 21 days. The cultures were filtered, lyophilised and kept at 4 °C until further use [9].

2.2. Bacterial strains

In the present study, 13 clinical isolates of *H. pylori* from antral biopsies were used (numbered PGH-189, PGH-219, PGH-220, PGH-229, PGH-259, PGH-607, PGH-841, PGH-2214, PGH-2215, PGH-2581, PGH-2613, PGH-2615, PGH-2722) and one reference strain (ATCC 43504).

2.3. Bacterial cultivation

All clinical isolates of *H. pylori* were obtained from the Endoscopic Unit – 1st Surgical Department of the Patision General Hospital (Athens, Greece) and submitted to the Department of Microbiology of the same Hospital into transport medium (Portagem pylori, BioMérieux, 42041). Primary isolation was performed on commercial selective Pylori agar (BioMérieux 43263) after incubation at 37 °C, under humid microaerobic atmosphere (Genbox microaer, BioMérieux, 96125) for 3–7 days. Following primary selective isolation, *H. pylori* strains were identified by usual diagnostic procedures, i.e., according to colony morphology, Gram-staining, biochemical tests [oxidase production test (APICAMPY, BioMérieux, 24800)] and molecular identification based on amplification of species specific sequences of 16S rRNA by polymerase chain reaction (PCR). For susceptibility testing, the strains were subcultured on Wilkins–Chalgren anaerobe agar (Oxoid, CM 619) supplemented with 8% horse blood (Oxoid, SR048C), 10% horse serum (Oxoid, CR035C) and 1% Vitox (Oxoid, SR090A). The *H. pylori* isolates were used immediately in the tests. A stock culture of each isolate was stored in Brain–Heart Infusion Broth (BioMérieux 51009) supplemented with 20% (v/v) glycerol (BDH) and 1% Yeast extract at −80 °C. It is noteworthy that *H. pylori* is an extremely demanding bacterium concerning its culture conditions. The number of the strains referred here is a part of the primary isolated strains.

2.4. Minimum inhibitory concentration (MIC) testing

The lyophilized mycelia were dissolved at 10.0 mg ml^{-1} with dimethyl sulfoxide (DMSO, Merck, Art. 2951) and diluted with nutrient medium (Brain–Heart Infusion Broth, BioMérieux 51009). Final concentrations of 250.0, 125.0, 62.5, 31.25, 15.6, 7.8, 3.9, 1.95 μg ml^{-1} were used. The proportion of DMSO never exceeded 1% in the medium [10]. The micro-dilution method was used.

Inocula of approximately 5×10^5 CFU were inoculated into Brain–Heart Infusion Broth (BioMérieux 51009) supplemented with 10% horse serum and 1% Vitox. The tests were performed on 96-well micro-titer plates cultured micro aerobically for 3 days at 37 °C in anaerobic jars. Dilutions of the inocula were sub cultured on Pylori Agar (BioMérieux 43263) to verify the absence of contamination and to check the validity of the inoculum. The MIC was taken as the lowest concentration of each fungus that inhibited visible growth (at the binocular microscope) after incubation period. DMSO was used as a control, while clarithromycin was used as a positive control. All experiments were conducted in duplicate.

3. Results and discussion

In screening natural resources for their antibacterial potential, it is reasonable to start from those organisms, which are known to possess such properties. The present study is oriented towards the exploitation of the biological properties of the secondary metabolites of micro-fungi and their application in human health. The inhibitory effect of the tested micromycetes on the growth of *H. pylori* is shown in Table 1. Of the 22 micromycetes tested, the most active were found to be *Penicillium ochlochloron* and *P. funiculosum* with MIC comparable to those of clarithromycin. So far, further investigation of the latter fungus allowed the isolation of the active metabolite (−) 2,3,4-trihydroxybutanamide with MIC ranging from 0.97–3.9 μg ml^{-1}.

3.1. Isolation and identification of the main active component

General experimental procedures. Optical rotation value was measured at 20 °C, in $CHCl_3$ (Uvasol) on a

Table 1
MIC (μg ml^{-1}) of micromycetes against *Helicobacter pylori* growth

Fungi		PGH-189	PGH-219	PGH-220	PGH-229	PGH-259	PGH-607	PGH-841	ATCC-43504
Alternaria alternata Fries, von Keissler	DSM 2006	–	–	–	–	–	–	–	–
Aureobasidium pullulans Arnaud de Bary	ATCC 9348	–	–	–	–	–	–	–	–
Candida albicans Robin	a	7.8	15.6	–	–	–	–	–	7.8
Chaetosphaeronema achaetosporioides Fresenius des Vries	ATCC 13276	7.8	15.6	15.6	–	15.6	31.2	31.2	7.8
Emericellopsis flaccosum Hatz, Langeron & Milodchevitch	a	–	15.6	15.6	–	15.6	–	–	–
Fabria fulicosa Cooke, Ciferri	TK 5318	–	–	15.6	–	15.6	31.2	–	–
Fusarium sporotrichioides Sherbakoff	ITM 499	–	–	–	–	–	–	–	–
Fusarium tricinctum Corda, Saccardo	CBS 51447	–	–	–	–	–	–	–	–
Microsporum canis Bodin	a	–	–	–	–	–	–	–	–
Mucor sp. Schipper 1978	b	–	–	–	–	–	–	–	–
Paecilomyces carlsii Bainier	DSM 1961	–	–	–	–	–	–	–	–
Penicillium funiculosum Thom	ATCC 36839	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9
Penicillium verrucosum Biourge	ATCC 9112	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9
Phoma exigua/*diversa* Wang and Zabel 1990	b	–	–	7.8	–	–	–	–	7.8
Phomopsis helianthi Muntanola-Cvetkovic, Mihaljcevic et Petrov	ATCC 201540	–	7.8	–	31.2	–	–	–	–
Rhizopus sp. Inui, Takeda, Ilzuka 1965	b	7.8	7.8	–	–	–	7.8	–	7.8
Stachybotrys chartarum Ehrenberg Fries S. Hughes	DSM 2144	–	–	–	–	–	–	–	–
Trichoderma viride Persoon, Fries	IAM 5061	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8
Trichophyton mentagrophytes Robin	a	–	–	–	–	–	–	–	–
Trichophyton rubrum Castell	a	15.6	–	–	–	–	–	–	–
Trichophyton tonsurans Malmsten	a	–	–	–	–	–	–	–	–
Trichosporon sp. Behrend, 1890	a	–	–	–	–	–	–	–	–
Clarithromycin		<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0

[a] Clinical isolates from patients.
[b] Isolates from Mycoteca of the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research, Belgrade.

Table 2
MIC (μg ml^{-1}) of (−) 2,3,4-trihydroxy-butanamide against *Helicobacter pylori* growth

	PGH-259	PGH-607	PGH-841	PGH-2214	PGH-2215	PGH-2581	PGH-2613	PGH-2615	PGH-2722	ATCC-43504
Compound	1.95	1.95	1.95	3.9	3.9	1.95	0.97	0.97	0.97	0.97
Clarithromycin	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0

Perkin–Elmer 341 polarimeter. IR spectra was obtained on a Perkin–Elmer Paragon 500 instrument. The ^{1}H NMR spectra (400 MHz) and ^{13}C NMR spectra (50.3 and 100.6 MHz) were recorded using Bruker DRX 400 and Bruker AC 200 spectrometers. Chemical shifts are reported in δ (ppm) values relative to TMS. COSY, HSQC, HMBC and NOESY (mixing time 950 ms) were performed using standard Bruker microprograms. High-resolution EI mass spectral data were recorded on a JEOL GCmate mass selective detector and were provided by the University of Notre Dame, Department of Chemistry and Biochemistry, Notre Dame, Indiana. Low-resolution EI mass spectral data were recorded on a HP 1100 MSD API-electrospray (using Na as reagent). Vacuum-liquid chromatography (VLC): silica gel (Merck; 43–63 μm). Column chromatography: Sephadex LH-20 (Pharmacia). HPLC: CE 1100 Liquid Chromatography Pump; Techsil 10 C_{18} (250 × 10 mm) column. Absorbents for TLC: Merck RP 18 F_{254s}, Art. 5685; Merck silica gel 60 F_{254s}, Art. 5554; Detection on TLC plates: UV light, anisaldehyde–H_2SO_4 spray reagent. Twenty-two grams of lyophilised mycelia of *P. funiculosum* were extracted into ultra-sonic bath with cyclohexane, dichloromethane and methanol, successively. The extracts were filtered and concentrated at low temperature. All extracts were tested for their anti-*H. pylori* activity. The methanol extract (1.8 g) was proved the most active and subjected to further bio-guided purification by VLC on silica gel (7.0 × 3.5 cm), using cyclohexane–ethyl acetate–methanol mixtures of increasing polarity as eluents to give 12 fractions of 300 ml each. Fraction H (cyclohexane–EtOAc, 75:25 – 25:75, 72.0 mg) was subjected to column chromatography on Sephadex LH-20 (35 × 4 cm; MeOH) and afforded 66 fractions of 10 ml each. After analytical TLC, similar fractions recombined to nine groups and tested against *H. pylori*. The active group D (fractions 25–46; 62.0 mg) was further subjected to reversed-phase HPLC (MeCN/H_2O 2:3, 1.9 ml min^{-1}) and allowed the isolation of (−) 2,3,4-trihydroxy-butanamide (7.0 mg; R_t 5.2 min). *(−) 2,3,4-trihydroxybutanamide*: white crystals; $[\alpha]_D^{20}$ −0.54° (c 0.05; H_2O); IR (KBr) ν_{max}: 2920.7–2850.5, 1260.4, 1028.8, cm^{-1}; HREI-MS *m/z* [M]$^+$ 135.0232, calcd. for $C_4H_9NO_4$ 135.0229. ^{1}H NMR (400 MHz, CD_3OD) δ: 3.77 (1H, d, J = 7.8, H-2), 3.69 (1H, dt, J = 3.3, 8.0, H-3), 3.81 (1H, dd, J = 10.9, 3.4, H-4a), 3.62 (1H, dd, J = 10.8, 5.8, H-4b); ^{13}C NMR (50.3 MHz, CD_3OD) δ: 71.7 (CH, C-2), 73.4 (CH, C-3), 66.5 (CH_2, C-4), 163.5 (-CO). MIC: Table 2.

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• BRIEF REPORTS •

Herpes simplex virus type 1 in peptic ulcer disease: An inverse association with *Helicobacter pylori*

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Abstract

AIM: To assess the frequency of herpes simplex virus type I in upper gastrointestinal tract ulcers and normal mucosa with the modern and better assays and also with a larger number of well characterized patients and controls and its relationship to *Helicobacter pylori*(H *pylori)*.

METHODS: Biopsy specimens from 90 patients (34 with gastric ulcer of the prepyloric area and 56 with duodenal ulcer) were evaluated. Biopsies from 50 patients with endoscopically healthy mucosa were considered as the control group. The method used to identify herpes simplex virus-1 (HSV-1) was polymerase chain reaction. *H pylori* was detected by the CLO-test and by histological method.

RESULTS: Herpes simplex virus-1 was detected in 28 of 90 patients with peptic ulcer (31%) [11 of 34 patients with gastric ulcer (32.4%) and 17 of 56 with duodenal ulcer (30.4%)] exclusively close to the ulcerous lesion. All control group samples were negative for HSV-1. The likelihood of *H pylori* negativity among peptic ulcer patients was significantly higher in HSV-1 positive cases than in HSV-1 negative cases ($P = 0.009$). Gastric ulcer patients with HSV-1 positivity were strongly associated with an increased possibility of *Helicobacter pylori* negativity compared to duodenal ulcer patients ($P = 0.010$).

CONCLUSION: HSV-1 is frequent in upper gastrointestinal tract ulcers but not in normal gastric and duodenal mucosa. There is an inverse association between HSV-1 and *H pylori* infection.

Key words: HSV-1; Herpes simplex virus type 1; Peptic ulcer; Duodenal ulcer; Gastric ulcer; PCR; Polymerase chain reaction; *H pylori*; Non-steroidal anti-inflammatory drugs

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INTRODUCTION

During the last two decades, a significant progress has been made in the role of *Helicobacter pylori*(*H pylori*) in the pathogenesis of peptic ulcers, while the invention of new powerful antisecretory drugs has changed dramatically the treatment of the disease. However, the exact etiopathogenesis of peptic ulcer disease is still under investigation.

The significant role of gastric acidity and inflammation of mucosa due to *H pylori* cannot be disputed, but a multifactorial etiology for peptic ulcer disease seems to be emerging[1-3].

The idea of a possible correlation between HSV-1 and peptic ulcers has appeared almost 40 years before[4,5], due to many common characteristics observed in the clinical picture and the natural history of both diseases[6,7].

A possible involvement of HSV-1 in peptic ulcer disease was reported from several investigators, but a firm conclusion has not yet been reached. The vast majority of these studies are based on the detection of antibodies against the virus in the serum and the duodenal juice of patients with peptic ulcer[8-12], a finding also common in the apparent healthy population. There are only two studies that report the presence of HSV-1 in tissue samples obtained from gastric and duodenal ulcers, using polymerase chain reaction (PCR) methods, but the number of the examined populations is small[13,14]. On the other hand, in the studies reported above, a possible correlation between HSV-1 and *H pylori* has been investigated in the

pathogenesis of peptic ulcer disease.

DNA of HSV-1 has been detected also in human vagal[15] and celiac ganglia[16], which provide the nerve network to gastric tissue. Theoretically, since vagotomy is used to treat peptic ulcer disease, the same treatment may interrupt the migration of activated HSV-1 from ganglia to gastric mucosa, thus preventing the recurrence of ulcer.

The purpose of this study was to investigate the possible relationship between HSV-1 and peptic ulcer and whether viral infection of ulcer patients is related to the presence of *H pylori* infection.

MATERIALS AND METHODS

Patients and biopsies

All patients who underwent esophagogastroduodenoscopy at our institution from September 1999 to September 2002 were recruited. The first group included 56 patients (31 men and 25 women) with active duodenal ulcer (average 53.5±15 years, range from 19 to 83 years). The second group included 34 patients (22 men and 12 women) with active ulcer of the prepyloric area of the stomach (mean of 61.5±16.2 years, range from 22 to 89 years) and the third group that formed the control group, consisted of 50 patients (28 men and 22 women) with no evidence of pathologic findings (mean of 54.8±16.7 years, range from 21 to 86 years).

Tissue samples were taken from all 90 patients with peptic ulcer for the detection of HSV-1 in duplicate, from the following areas: the base and the rim of the ulcer; the adjacent area of the ulcer at a distance of 3 cm (minimal and maximal distance from the crater 3 and 5 cm respectively). For this reason, in the duodenal ulcer group a second duodenal biopsy was obtained; an endoscopically normal area of the stomach (the corpus in gastric ulcer cases and the antrum in duodenal ulcer cases).

Two samples were also taken from endoscopically healthy areas of the antrum and corpus of the stomach in all 50 controls.

Two samples from the antrum and two from the corpus of the stomach were taken for the detection of *H pylori* using the rapid urease test (CLO-test) and routine histology.

Finally, specimens from the gastric ulcers were examined histopathologically to exclude malignancy.

Risk factors probably involved in the pathogenesis of peptic ulcer, such as non-steroidal anti-inflammatory drugs (NSAIDs), smoking, alcohol, history of herpes labialis, family history of peptic ulcer, and ulcer site, were recorded and analyzed.

DNA extraction

Genomic DNA was extracted using the QIAamp DNA mini kit, following the protocol supplied for purification from fresh tissues. DNA was finally dissolved in 50-100 μL of TE buffer depending on the size of DNA pellets and stored at -20 °C until amplification.

Primer design

For the nested PCR assay, oligonucleotides deduced from the published sequence of the RL2 gene-coding region from HSV-1 were used[17]. For the control DNA assay, oligonucleotides for b-actin gene were used. The primer sequences and characteristics are shown in Table 1.

Table 1 Characteristics and nucleotide base sequences of primers used for nested PCR and control assays

Gene target	GenBank accession number	Product size (bp)	Sequences[a]	T_m (°C)
HSV-1 RL2	X14112	450	Outer sense agcagcgacctcgagcgcgacg	69.1
			Outer antisense tgcgggtccgcgcgtcgtcaga	71.4
		110	Inner sense ccggcagtcgcgggcgc	73.4
			Inner antisense aaggtgtcgcagcggcaggtg	69.8
b-actin		200	Forward gtgatctccttctgcatcc	53.2
			Reverse ctctccagccttcctc	52.7

T_m, melting temperature. [a]Sequences shown are in the 5′ to 3′ direction.

Nested PCR amplification and detection assay of HSV-1

B-actin PCR generating a 200-bp product was performed to determine the DNA integrity of the samples. For the quality control PCR assay, the following program was used 1 cycle at 94 °C for 2 min; 35 cycles at 94 °C for 30 s, at 58 °C for 30 s, at 72 °C for 30 s; and a final cycle at 72 °C for 7 min.

PCR detection of HSV-1 was carried out in a 50 μL reaction mixture containing 25 μL of Taq PCR master mix solution (Qiagen), 13 μL of double-distilled DNase-free water, 1 μmol/L concentration of each primer and 10 μL of the extracted sample. PCR was performed on a PE 9 600 thermocycler (Perkin-Elmer Cetus, Branchburd, NJ, USA). The cycling conditions were at 94 °C for 1 min, 5 cycles at 94 °C for 5 s and at 72 °C for 4 min; 5 cycles at 94 °C for 5 s and at 70 °C for 4 min; 30 cycles at 94 °C for 5 s and at 68 °C for 4 min. After the final cycle, tubes were incubated for an additional 10 min at 72 °C. Nested PCR amplification was done with a 0.5 μL aliquot from the first run, 25 μL of Taq PCR master mix solution, 18 μL of double-distilled DNase-free water and 1 μmol/L concentration of each inner primer under the following cycling conditions: at 94 °C for 2 min; 35 cycles at 90 °C for 30 s, at 68 °C for 30 s, at 72 °C for 30 s and a final extension at

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72 ºC for 10 min. Each amplification run contained one negative and one positive control. The negative control consisted of blank reagent and water. For the positive control, HSV-1 genomic DNA provided by Sigma was used. Consistent PCR analyses were repeated twice or more.

The PCR products were analyzed by 2% agarose gel electrophoresis in 0.5×Tris–borate EDTA buffer along with ethidium bromide. A molecular weight marker (Φx 174/Hae III, Sigma) was also run simultaneously to identify the molecular size of the PCR products. The DNA bands were visualized by UV transillumination and analyzed using a gel-documentation system. None of the PU and the control samples were negative in the b-actin test ultimately leaving 90 PU and 50 controls that were subjected to HSV-1 PCR analysis.

Helicobacter pylori testing

For the detection of *H pylori*, a CLO-test was used with high sensitivity and specificity (Kimberly-Clark CLO test, Ballard Medical products, Draper, UT 84020, USA)[18,19].

An experienced pathologist also assessed the histological sections with Giemsa stain[20].

Statistical analysis

All associations between parameters of interest were examined either by Fisher's exact test or Pearson's *chi square* test with continuity correction.

Multivariate analysis was performed using the stepwise logistic regression model to assess the contribution of the common risk factors to peptic ulcer development and *H pylori* detection.

$P<0.05$ was considered statistically significant.

RESULTS

Polymerase chain reaction

The genome of HSV-1 was present in 28 of the 90 patients with peptic ulcer (31.1%), contrary to the control group in which no positive detections were found (0%, $P<0.0005$, Figures 1-3). There was an equal prevalence in the two subgroups of patients, 17 of 56 patients with duodenal ulcer (30.4%) and 11 of 34 with gastric ulcer (32.4%) were tested positive ($P = 0.843$). In all HSV-1 positive cases, the viral genome was detected from the tissue samples obtained from the crater of the ulcer as from the samples obtained from the rim, while all samples from adjacent and distant areas were negative.

CLO test

All the *H pylori* positive subjects by CLO test from both groups were also positive for the bacteria with histology.

The incidence of *H pylori* was significantly higher in peptic ulcer patients (76/90, 84.4%) than in controls (30/50, 60%) ($P = 0.002$). A statistically significant difference was also found between patients with duodenal ulcer and those with gastric ulcer ($P = 0.036$). Negative *H pylori* was more frequently observed in patients with gastric ulcer (26.5%) than in patients with duodenal ulcer (8.9%, Table 2).

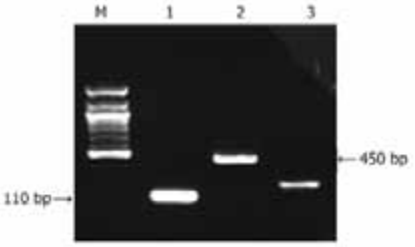

Figure 1 Nested PCR with HSV-1 outer and inner primers (450- and 110-bp amplicons; arrows) on 2% agarose gel, by ethidium bromide staining. Lane M: DNA molecular weight marker Φ×174/Hae III; lane 1: HSV-1 positive control (second run of nested PCR); lane 2: HSV-1 positive control (first run of nested PCR); lane 3: b-actin quality control PCR peptic ulcer sample.

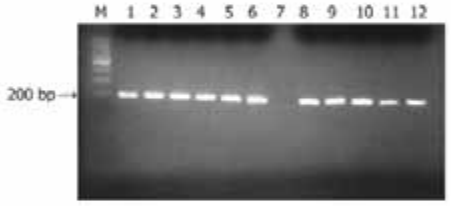

Figure 2 PCR quality assay control. Lane M: DNA molecular weight marker Φ ×174/Hae III; lanes 1-6: positive b-actin peptic ulcer samples; lane 7: negative control (without template); lanes 8-12: positive b-actin peptic ulcer samples.

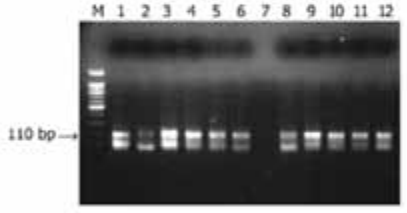

Figure 3 Nested PCR amplification of HSV-1 in samples of patients with peptic ulcer. Lane M: DNA molecular weight marker Φ×174/Hae III; lane 1: HSV-1 positive control (HSV-1 genomic DNA by Sigma); lanes 2-6: positive samples from patients with peptic ulcer; lane 7: negative control (without template); lanes 8-11: positive samples from patients with peptic ulcer; lane 12: HSV-1 positive control.

PCR and CLO test were significantly associated with respect to *H pylori* detection in all 90 peptic ulcer patients. Negative *H pylori* was more frequently detected in positive PCR samples (32.1%) than in negative PCR samples (8.1%) ($P = 0.009$) (Table 2). In the group of duodenal ulcer patients, *H pylori* negativity was more frequently observed in positive PCR samples (11.8%), than in negative PCR samples (7.7%). However, this difference was not

Table 2 Association between *H pylori* and HSV-1 in patients with peptic ulcer

	H pylori (+), %	*H pylori* (-)	*P*	Odds ratio (95%CI)
Peptic ulcer	76/90 (84.4)	14/90		
Controls	30/30 (60)	20/30	0.002	3.7 (1.6-8.1)
Gastric ulcer	23/34 (73.5)	9/34		
Duodenal ulcer	51/56 (91.1)	5/56	0.056	5.67 (1.1-12.11)
HSV-1 (+)	19/28 (67.9)	9/28		
HSV-1 (-)	57/62 (91.9)	5/62	0.009	5.4 (1.61-18.11)
HSV-1 (+)/GU	4/11 (36.4)	7/11		
HSV-1 (-)/ GU	21/23 (91.3)	2/23	0.002	18.37 (2.79-122.94)
HSV-1 (+)/ Duodenal ulcer	15/17 (88.2)	2/17		
HSV-1(-)/ Duodenal ulcer	36/39 (92.3)	3/39	0.634	-

HSV-1 (+)/gastric ulcer, patients with gastric ulcer positive for HSV-1; HSV-1 (-)/gastric ulcer, patients with gastric ulcer negative for HSV-1; HSV-1 (+)/duodenal ulcer, patients with duodenal ulcer positive for HSV-1; HSV-1 (-)/duodenal ulcer, patients with duodenal ulcer negative for HSV-1; CI, confidence interval.

Table 3 Association between *H pylori* and HSV-1 in relation with the site of peptic ulcer

	H pylori (+), %	*H pylori* (-)	*P*-value	Odds ratio (95% CI)
HSV-1(+)/ gastric ulcer	4/11 (36.4)	5/11	0.010	13.13 (1.92-89.5)
HSV-1(-)/ duodenal ulcer	15/17 (88.2)	2/17		

HSV-1 (+), patients positive for HSV-1; CI, confidence interval.

statistically significant ($P = 0.634$) (Table 2) whereas it was statistically significant in the subgroup of gastric ulcer patients ($P = 0.002$). In the gastric ulcer subgroup, *H pylori*, negative cases were observed in 63.6% of positive PCR samples and in 8.7% of negative PCR samples (Table 2). Finally, the likelihood of negative *H pylori* in HSV-1 positive samples in the group of gastric ulcer patients was significantly higher than that in the group of duodenal ulcer patients ($P = 0.010$) (Table 3).

Statistical analysis of other parameters

We also studied some of the common risk factors for the development of peptic ulcer disease. No statistically significant difference was found between patients and controls regarding family history of upper gastrointestinal ulcer, history of *Herpes labialis*, alcohol consumption, and use of NSAIDs (Table 4).

As expected, tobacco smoking was the only statistically significant risk factor for the development of peptic ulcers between patients and control population ($P = 0.019$). Smokers were associated with a 2.57-fold increase risk of peptic ulcer development.

The performed multivariate analysis also confirmed that *H pylori* and tobacco smoking (OR: 3.320 and 2.619 respectively) were more likely to induce peptic ulcer (Table 5).

Table 4 Risk factors and peptic ulcer disease

	Peptic ulcer patients (%)	Controls (%)	Odds ratio	*P*
History of *H labialis*				
Yes	24/90 (26.7)	9/50 (18.0)	-	0.302
No	66/90 (73.3)	41/50 (82.0)		
Alcohol consumption				
Yes	52/90 (55.6)	14/50 (28.0)	-	0.469
No	58/90 (64.4)	36/50 (72.0)		
NSAID use				
Yes	23/90 (25.6)	8/50 (16.0)	-	0.275
No	67/90 (74.4)	42/50 (84.0)		
Smoking				
Yes	45/90 (50.0)	14/50 (28.0)	2-57	0.019
No	45/90 (50.0)	36/50 (72.0)		
Family history of peptic ulcer				
Yes	30/90 (33.3)	9/50 (18.0)	2.5	0.076
No	60/90 (66.7)	41/50 (82.0)		

Table 5 Contribution of other risk factors to peptic ulcer development

	B	S.E	df	Sig.	Odds ratio	95%CI for Odds ratio
Smoking	0.963	0.394	1	0.015	2.619	1.209-5.673
H pylori infection	1.2	0.473	1	0.011	3.32	1.313-8.389
Age	0.011	0.014	1	0.432		
Sex	0.144	0.442	1	0.743		
History of *H labialis*	0.721	0.484	1	0.137		
Alcohol	0.021	0.492	1	0.966		
NSAID use	0.286	0.494	1	0.562		
Family history	0.44	0.49	1	0.309		
Constant	-0.741	0.393	1	0.06		

CI, confidence interval.

DISCUSSION

Despite the progress during the last 20 years in the understanding of the pathogenesis of peptic ulcer disease, it is clear that gastroduodenal ulcer is the result of a multifactorial process.

H pylori infection and NSAIDs have been recognized as the two most important causes of peptic ulcer disease. The proportion of peptic ulcers not associated with *H pylori* infection or the use of NSAIDs is increasing. Yet several studies have shown that 4.1-44% of peptic ulcers are not related to either of the two factors[21-25].

The possible involvement of HSV-1 in the process is a field of interest for several investigators, but a firm conclusion has not been reached. The presence of viral DNA in tissue samples obtained from the ulcer site is 9.5-18%[11,14]. The possible explanation for this finding is that the HSV-1 expression is prompted either by the ulcer injury or by immune cells[14,26] or HSV-1 itself might cause the ulcerative lesion by directly infecting the mucosal cells[15] or finally that HSV-1 expression is induced by the ulcer treatment[26-29].

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In the present study, in a substantially larger number of patients than in the studies reported above (90 *vs* 22 and 21 respectively), the positivity for HSV-1 DNA was expressed using PCR in a greater percentage of samples (31% *vs* 9.5% and 18% respectively).

It should be noted that viral DNA was detected only in the tissue samples obtained from the base and the rim of the lesions, whereas all the other examined samples obtained from the adjacent ulcer areas and endoscopically healthy mucosa from the patients and the control group were negative for the viral genome.

This finding can be explained as follows. The HSV-1 may have initially entered the vagus ganglia through the oral pharynx or other peripheral connecting sites. Upon activation, the virus would travel down the vagal nerve to the potential site of peptic ulcer lesion. HSV-1 itself might cause ulcerative lesion in selected cases of a subset of peptic ulcer diseases by directly infecting the mucosal cells in the stomach and duodenum following virus release from neuroendocrine cells or vagal nerve terminals or both. Alternatively, peptic ulcer might activate latent HSV-1 in vagal ganglia, making replication of HSV-1, a contributing but not an initiating factor of ulcer[33].

The detection percentage of positive HSV-1 was similar between the group of patients with gastric and the group of patients with duodenal ulcer (32.4% and 30.4% respectively), in contrast to previous studies, where the viral DNA is demonstrated only in tissue samples from gastric ulcers[13,14]. The exact cellular localization of HSV-1 DNA could not be identified.

On the other hand, investigating a possible association between *H pylori* and HSV-1 in pathogenesis of a subset of gastroduodenal ulcers, our data suggest that the PCR HSV-1 positivity is associated with a 5.4-fold increase in negative *H pylori* detection. Moreover, the patients with ulcer lesions infected with HSV-1 presented a similar prevalence of *H pylori* infection as the control group, which was significantly lower than that in the HSV-1 negative ulcer cases ($P = 0.09$). This finding requires further investigation.

Possible interpretations for the increased HSV-1 DNA positive detection rate in *H pylori* negative ulcers include the following. *H pylori* negativity is influenced by the viral expression, HSV-1 negativity influences *H pylori* infection and the virus independently causes some gastroduodenal ulcers.

According to our results, in the subgroup of patients with duodenal ulcers, the risk of *H pylori* infection was independent from HSV-1 DNA expression ($P = 0.634$). On the other hand, in the subgroup of patients with gastric ulcer disease, the possibility of *H pylori* negativity was 18.5-fold higher ($P = 0.002$). These data are in accordance with those reported by Lohr *et al*[13].

Additionally, according to our results, there was not any correlation between peptic ulcer disease and age, sex, ulcer site, family history of gastroduodenal ulcers, history of *H. labialis*, alcohol consumption and NSAIDs use. On the contrary, statistically significant difference ($P = 0.019$) was observed between patients with peptic ulcers and controls, as far as smoking was concerned.

Our results indicate an involvement of HSV-1 in the pathogenesis of peptic ulcer disease. Although an opportunistic infection with the virus in the ulcer site cannot be excluded, the inverse relationship between HSV-1 detection and *H pylori* infection indicates a possible implication of this virus in the formation of the ulcer crater, at least in a subgroup of patients. Furthermore, experimental data support this[13,15,16,30,31]. The exact localization of the virus in ulcer tissue cells should be precisely determined in order to clarify whether the lesion is caused by HSV-1 or the virus opportunistically is established, especially in immunocompromised patients[32].

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• RAPID COMMUNICATION •

Retinol-binding protein, acute phase reactants and *Helicobacter pylori* infection in patients with gastric adenocarcinoma

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Abstract

AIM: To determine the serum levels of c-reactive protein (CRP), transferrin (TRF), a2-macroglobulin (A2M), ceruloplasmin (CER), a1-acid glycoprotein (AAG), pre-albumin (P-ALB) and retinol-binding protein (RBP) in gastric carcinoma patients and to explore their possible correlation with underlying *Helicobacter pylori* (*H pylori*) infection.

METHODS: We measured the serum levels of CRP, TRF, A2M, CER, AAG, P-ALB, and RBP in 153 preoperative patients (93 males; mean age: 63.1±11.3 years) with non-cardia gastric adenocarcinoma and 19 healthy subjects.

RESULTS: The levels of CRP, CER, RBP, and AAG in cancer patients were significantly higher than those in healthy controls (P<0.0001), while no difference was found regarding the TRF, P-ALB, and A2M levels. Cancer patients with *H pylori* infection had significantly lower RBP values compared to non-infected ones (P<0.0001) and also higher values of CRP and AAG (P = 0.09 and P = 0.08, respectively).

CONCLUSION: High serum levels of CRP, CER and AAG in cancer patients do not seem to be related to *H pylori* infection. Retinol-binding protein seems to discriminate between infected and non-infected patients with gastric carcinoma. Further studies are needed to explore if it is directly involved in the pathogenesis of the disease or is merely an epiphenomenon.

Key words: Gastric cancer; *Helicobacter pylori*; Acute phase reactants; A1-acid glycoprotein; Transferrin; A2-macroglobulin; Ceruloplasmin; Retinol-binding protein; Pre-albumin; c-reactive protein

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INTRODUCTION

Though the incidence of gastric adenocarcinoma has decreased during the last 50 years, it still remains one of the most common cancers worldwide. Even though the exact molecular mechanisms leading to gastric carcinogenesis are incompletely understood, the current school of thought accepts a multifactorial model in which various dietary and non-dietary factors (i.e. *Helicobacter pylori* infection) operate at different steps in the process.

Epidemiological studies in the early 1990s demonstrated an up to sixfold increased risk of developing gastric adenocarcinoma in patients infected with *H pylori*[1-5]. More than 10 years ago, *H pylori* was classified as a type I carcinogen for human beings by the World Health Organization, being recognized in this way as a crucial player in the gastric carcinogenesis. Increased epithelial cell proliferation and oxidative damage of the gastric mucosa are the two main mechanisms that seem to operate as a result of *H pylori* infection[6].

Table 1 Characteristics of studied populations and demographic data

Group	Number of subjects	Sex (M/F)	Mean age(yr)	Tumor location (antrum/body)
Gastric cancer	153	93/60	63.1	125/28
Infected	82	46/36	64.2	64/18
Non-infected	71	42/29	62.4	56/15
Healthy controls	19	10/9	62.2	

M, male; F, female.

The other environmental factor that has been shown to play a significant role in gastric carcinogenesis is diet. Diets with low intake of fruits, vegetables and milk and high intake of smoked or salted foods, dried fish, cooking oil, and complex carbohydrates have been shown to increase the risk for gastric cancer[5-7]. Contrary to that, intake of antioxidants has been associated with decreased risk of gastric cancer[8].

The search for useful biomarkers that can help in the diagnosis of cancer and add prognostic information to that already provided by the tumor staging is a field of active research. Even though numerous studies have been published, no single substance has been found to be clinically useful in the management of patients with gastric carcinoma. Immunohistochemical analysis of gastric tumors has shown that they contain protease inhibitors such as a2-macroglobulin[9,10] or a1-acid glycoprotein[11-13] and suggests that they may serve as markers of tumor aggressiveness. Some data also suggest that the acute phase reactants CRP and AAG may have prognostic value[13,14]. Stored sera from patients that later develop gastric cancer contain lower values of ferritin, while transferrin values were not different[15]. Ceruloplasmin has been shown to be useful in some studies[16] but not in others[17-17]. Serum interleukin-6 (IL-6) level correlates with the disease status of gastric cancer, suggesting that it may be used as a new tumor marker for monitoring the response to treatment[18]. Other studies even suggested that IL-6 might have a direct role in the pathogenesis[19,20]. A recent study[21] confirmed that IL-6 is a useful parameter for the diagnosis and grading of gastric cancer, but also suggested a role for malonyldialdehyde (MDA), nitric oxide (NO) and, especially vascular endothelial growth factor (VEGF). Finally, inconclusive data surround the usefulness of the so-called negative acute phase reactants, pre-albumin, and retinol-binding protein, in the assessment of cancer patients[22-26]. Retinol-binding protein was also included in our analysis because retinol and its ligand has been associated with lower risk of gastric cancer in some studies[27] but not in others[28].

The aim of this prospective, observational study was to measure the serum levels of the acute phase reactants a1-acid glycoprotein, transferrin, a2-macroglobulin, ceruloplasmin, retinol-binding protein, pre-albumin, and c-reactive protein in patients with gastric adenocarcinoma and to explore their correlation with *H pylori* infection.

MATERIALS AND METHODS

Patients

One hundred and fifty-three patients (93 men, 60 women) with a mean age of 63.1±11.3 years and histologically confirmed non-cardia gastric cancer were included in the study. In each case we recorded the location of the tumor, the histological type and the lymph node involvement. One hundred and twenty-five malignant tumors were located in the antrum and 28 in the body of the stomach. None of the patients had any gross metastatic disease as determined by chest and abdomen CT scans and received chemotherapy or radiation therapy prior to surgery. The histological diagnosis was based on morphologic examination of hematoxylin/eosin-stained specimens. Table 1 summarizes the demographic data and characteristics of the studied populations. Nineteen healthy volunteers (10 men, 9 women) with a mean age of 62.2±13.1 years were used as controls. We defined "healthy" status as the absence of a cardiovascular disorder, malignancy, gastrointestinal pathology, and *H pylori* infection. Neither patients nor controls had any evidence of infection or received antibiotics for at least 2 mo prior to serum collection with only the use of antacids. Sera from 48 patients were prospectively collected, the rest of the sera were collected in the previous 17 mo and kept frozen at -70 ℃.

The study was approved by the Institutional Review Boards of the participating hospitals and all study individuals gave their informed consent.

Determination of H pylori serology

All enrolled subjects (cancer patients and controls) underwent an enzyme-linked immunosorbent assay (ELISA) IgG serologic test for *H pylori* (Allergy Immunotechnologies Inc., Newport Beach, CA, USA) in accordance with the manufacturer's instructions. The specificity and sensitivity of the serology test validated in our local population were 95% and 90%, respectively. *H pylori* antibody titers higher than 155 mU/L were considered positive and lower than 155 mU/L negative.

Determination of acute phase proteins

Concentrations of the specific acute-phase proteins (c-reactive protein, a1-acid glycoprotein, ceruloplasmin, transferrin, a2-macroglobulin, prealbumin, and retinol-binding protein) were measured by nephelometric method on a Dade Behring nephelometer BNII (Dade Behring, USA), using Dade Behring antibodies and standard reagents. The intra- and inter-assay coefficients of variation were in the range of 2% and 5%, respectively.

Examined parameters

Serum levels of the acute phase reactants were recorded from healthy controls and patients suffering from gastric cancer. These patients were grouped according to whether they were infected with *H pylori* or not. Normal values for the examined parameters were as follows: c-reactive protein (CRP) <50 mg/L, A1-acid glycoprotein (AAG) <1

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Table 2 Mean levels of TRF, A2M, AAG, CER, RBP, P-ALB, and CRP in cancer patients and controls

Parameter (mg/dL)	Cancer patients (n = 153)	Controls (n = 19)	P
TRF	265.43	274.95	NS
A2M	263.28	253.11	NS
AAG	245.48	94.79b	<0.001
CER	53.26	41.74b	<0.001
RBP	23.78	3.77b	<0.001
P-ALB	23.04	17.74	NS
CRP	9.32	2.92b	<0.001

bP<0.001 vs controls; NS: not significant.

Table 3 Mean levels of TRF, A2M, AAG, CER, RBP, P-ALB, and CRP between infected and non-infected cancer patients

Parameter (mg/dL)	Non-infected (n = 71)	Infected (n = 82)	P
TRF	276.46	255.88	NS
A2M	281.49	247.46	NS
AAG	238.21	251.77	NS
CER	52.03	54.32	NS
RBP	31.89	16.75b	<0.001
P-ALB	22.35	23.64	NS
CRP	7.99	10.47	NS

bP<0.001 vs infected; NS: not significant.

200 mg/L, ceruloplasmin (CER) <550 mg/L, transferrin (TRF) <4 300 mg/L, A2-macroglobulin (A2M) <3 200 mg/L, pre-albumin (P-ALB) <450 mg/L and retinol-binding protein (RBP) <60 mg/L.

Statistical analysis

The nonparametric Mann-Whitney *U*-test was employed to study the differences in values between cancer patients and controls and to compare the significance of values' differences between infected and non-infected patients. $P<0.05$ was considered statistically significant. All analyses were completed using the Statistical Package for Social Sciences (SPSS 11.5).

RESULTS

The assessed parameters found between cancer patients and healthy controls and between infection and non-infection groups of cancer patients are shown in Tables 2 and 3, respectively. Out of a total of 153 cancer patients participating in the study, 82 (53.6%) were infected with *H pylori*. The levels of CRP, CER, RBP, and AAG in cancer patients were significantly higher than those in healthy controls ($P<0.0001$), while no difference was found regarding the TRF, P-ALB, and A2M levels. Cancer patients with *H pylori* infection had significantly lower RBP ($P<0.0001$) and higher CRP and AAG ($P = 0.09$ and $P = 0.08$, respectively) than those without *H pylori* infection.

DISCUSSION

In the current study, we have measured the serum levels of a group of acute phase reactants in patients with adenocarcinoma localized in the non-cardia part of the stomach. We separated these patients to either antibody-positive or antibody-negative to *H pylori*. From the examined acute phase reactants, we found that the levels of c-reactive protein, ceruloplasmin, a1-acid glycoprotein and retinol-binding protein in cancer patients were significantly higher than those in healthy controls, while no difference was found regarding the transferrin, prealbumin and a2-macroglobulin levels. However, only retinol-binding protein was significantly related to *H pylori* infection as its values were significantly lower in infected patients than in non-infected ones. The etiology of these biochemical aberrations is probably multifactorial.

Numerous studies have shown that gastric cancer patients have significantly higher levels of CRP[19,20] than healthy controls. A previous study[19] has even showed that elevated CRP levels have a prognostic significance and a recent study suggested that CRP levels contribute to the diagnosis of infection in cancer patients[21]. Our data confirm that CRP is elevated in cancer patients, but we cannot comment on its usefulness in the diagnosis of infection in cancer patients, as this was not the aim of our study. Though it was not different between infected and non-infected patients with *H pylori* ($P = 0.09$), one possible explanation is that chronic *H pylori* infection did not cause the elevation of CRP with acute infections as pneumonia or bacteremia. The other explanation is that our study did not have the statistical power to detect any difference.

Immunohistochemical analysis of the tumor epithelium showed that the protease inhibitor a2-macroglobulin is related to the invasive growth of gastric cancers[9,10]. Similar preliminary data exist for a1-acid glycoprotein[11-13]. Our data do not support routine measurement of A2M, but confirmed that AAG is a potentially useful marker as it was consistently higher in cancer patients and showed a trend to reach statistical significance in *H pylori*-infected patients ($P = 0.08$).

The role of iron-carrying protein transferrin is undetermined. Studies showed that there is no difference in the mean TRF values between controls and patients while ferritin values are significantly different[14,22], suggesting that TRF may not be a prognostic factor for future gastric carcinogenesis but its role remains uncertain. In accordance with previous studies, our data did not require measuring transferrin in gastric cancer patients whether they were infected with *H pylori* or not.

Finally, we decided to include the so-called nutritional indices (prealbumin and retinol-binding protein) in our analysis to explore their relation to cancer or *H pylori* infection. In patients with colon cancer, the levels of serum prealbumin, retinol-binding protein, transferrin, and albumin were interrelated and tended to show a similar pattern of change. More specifically, in metastatic colon cancer, prealbumin was the most sensitive indicator of nutritional status and its levels and rates of change

had a prognostic significance. A rapid fall of prealbumin often occurs 2-3 mo prior to death of the patients and this preterminal phase is also frequently heralded by a progressive rise in the CRP level in the absence of any obvious infection[36]. Prealbumin concentration has a prognostic importance in women with epithelial ovarian carcinoma[37] and a general cancer population as well[37]. We cannot confirm the data on the usefulness of prealbumin testing because we did not perform any formal assessment of nutritional status in our study population.

Serum ceruloplasmin levels are higher in gastric and lung cancer[38] and our data have confirmed this finding. We did not find any correlation of *H pylori* infection with ceruloplasmin levels and cannot suggest a pathogenetic role for this copper-chelating protein apart from being a marker of systemic inflammation.

The most interesting findings of our study are the significantly higher levels of retinol-binding protein in gastric cancer patients than healthy controls, the only marker being statistically different between infected and non-infected patients with *H pylori* (it was lower in *H pylori*-infected patients). Our findings are novel and in contrast with previous studies showing decreased RBP levels in the lung[39] and colorectal cancer[17]. Retinol, the ligand of retinol-binding protein, is required to maintain immunity and epithelial turnover and is a key micronutrient needed for combating infection. Studies have shown a good correlation between RBP and retinol even in the context of infection and protein malnutrition[39]. Retinol deficiency could either directly disrupt epithelial integrity or indirectly increase susceptibility to the damaging factors contained in either tobacco smoke (in the case of lung cancer) or diet (in the case of gastric and colorectal cancer). One would expect a lower and not a higher retinol-binding protein value in gastric cancer patients.

A fundamental difference between stomach cancer and other types of cancer is the involvement of *H pylori* in the former. There is evidence that *H pylori* infection *per se* is associated with the abnormalities of the nutritional markers even in the absence of malignancy. Aguilera *et al*.[32] studied the relationship between *H pylori* infection, anorexia and malnutrition in 48 peritoneal dialysis patients and found that infected patients with anorexia have lower lymphocyte counts, pre-albumin, transferrin, serum albumin, normalized equivalent of protein-nitrogen appearance and residual renal function. Eradication of *H pylori* could significantly improve the clinical syndrome and the biochemical abnormalities implying a causative role, but unfortunately retinol-binding protein was not measured in that study. The significant decrease in the activity of class IV alcohol dehydrogenase (ADH) in the antrum and corpus of stomachs of men and women infected with *H pylori* may be one of the underlying mechanisms as class IV ADH is the major isoenzyme controlling the production of retinoic acid from retinol and its supply to the human gastric mucosa[33,34]. It has been shown in animal models that the inflammatory response to infection and tissue injury is associated with low concentrations of plasma retinol and its specific transport proteins, retinol-binding protein and pre-albumin[35], suggesting that inflammation-induced hyporetinemia is attributed to a reduction in the hepatic synthesis of RBP and secretion of the retinol-RBP complex. The marked depressing effect of infections on serum retinol and retinol-binding protein has also been shown in human beings[16,17]. We speculate that *H pylori* infection in gastric carcinoma patients can lead to a decrease of retinol-binding protein by one of the above mechanisms but it remains unclear why uninfected patients with gastric cancer still have higher RBP levels than healthy controls.

The role of retinoids in gastric carcinogenesis has been studied in epidemiologic studies with conflicting results[27,28], but the molecular mechanisms that operate at either healthy or disease states have begun to be elucidated. Retinol has been shown to enhance differentiation of the gastric cell lineage in developing rabbits[29] and the reduction of retinoic acid signal has been implicated in the development and evolution of pre-malignant lesions of the human gastric mucosa[30]. The activation of the retinoic acid receptor induces cell differentiation and may antagonize cancer progression. Cellular retinol-binding protein I (CRBP-I) functions in retinol storage and its expression is lower in human cancers than in normal cells. A very recent study[30] showed that CRBP-I downregulation in human mammary epithelial cells chronically compromises retinoic acid receptor activity, leading to the loss of cell differentiation and tumor progression. The fact that class IV ADH is the major isoenzyme responsible for retinoic acid production from retinol and the more significantly decreased enzyme in the presence of *H pylori* infection associated with morphologic changes in the human gastric mucosa is intriguing. The retinoic acid pathway may be one of the missing links in the interplay of *H pylori* infection with gastric carcinogenesis.

In conclusion, gastric cancer patients with *H pylori* infection have significantly lower retinol-binding protein values than non-infected ones and both infected and non-infected groups have higher retinol-binding protein values than healthy controls. This finding may add to our understanding and management of this dreadful disease.

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Medical Hypothesis

A concept on the role of *Helicobacter pylori* infection in autoimmune pancreatitis

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- **Introduction**
- **Definition and terminology of AIP**
- **Pathophysiology / pathogenesis of AIP**
- **Apoptosis and autoimmunity**
- ***Helicobacter pylori*, autoimmunity and apoptosis**

Abstract

Autoimmune pancreatitis, an inflammatory process of the pancreas due to an autoimmune mechanism establishing etiology of chronic pancreatitis, is characterized by the presence of autoantibodies, hypergammaglobulinemia, pancreatic enlargement, pancreatic duct strictures, and pathologic features of fibrotic changes with intense, mainly lymphocytic infiltrations, which may contribute to tissue destruction probably by apoptosis. In almost 60% of the cases, this type of pancreatitis coexists with other autoimmune diseases such as Sjögren's syndrome, sclerosing extrahepatic cholangitis, primary biliary cirrhosis, autoimmune hepatitis, or other extrapancreatic disorders, and recently with gastric peptic ulceration. The diversity of extrapancreatic lesions with similar histopathologic findings suggests general involvement of the digestive system in this disease, although the presence of such involvement has not been fully elucidated. Similarly, *Helicobacter pylori (H. pylori)* infection, a well known cause of gastric ulcer, has been associated, via molecular mimicry of host structures by its constituents with the same autoimmune conditions, also characterized by fibrotic changes and/or lymphoplasmacytic inflammations, accompanied by aberrations of T cell apoptosis that contribute to hepatobiliary- or extrahepatic-tissue destruction. Considering that *H. pylori* is involved in the pathogenesis and pathophysiology of these autoimmune disorders, we propose that this organism might trigger autoimmune pancreatitis through induction of autoimmunity and apoptosis.

Keywords: autoimmune pancreatitis - *Helicobacter pylori* - molecular mimicry - apoptosis - T cells

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Introduction

Since Sarles *et al* [1] observed a case of specific pancreatitis associated with hypergammaglobulinemia that was not associated with alcohol consumption, subsequent reports have described patients with chronic pancreatitis characterized by the presence of autoantibodies, elevated levels of immunoglobulins (IgG4), enlargement of the pancreas (diffuse or focal), pancreatic duct strictures, and pathologic features of fibrotic changes with an intense inflammatory cell (mainly lymphocytic) infiltration [2-6]. These major lymphocytic infiltrations may contribute to tissue destruction probably by apoptosis [7-10]. In approximately 60%, the coexistence of this type of pancreatitis with other autoimmune diseases such as Sjögren's syndrome (SjS), sclerosing extrahepatic cholangitis [interpreted as a variant of primary sclerosing cholangitis (PSC) or as an inflammatory pseudotumor], primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH), or other extrapancreatic disorders, such as retroperitoneal fibrosis, salivary gland swelling, inflammatory bowel disease (IBD), Hashimoto's thyroiditis, and recently gastric peptic ulceration has been reported [4-6,11]. The histopathologic findings in these extrapancreatic lesions are lymphoplasmacytic inflammation and fibrosis, similar to those in the pancreatic tissue, suggesting a common pathogenesis [12-17]. The diversity of extrapancreatic lesions with similar histopathologic findings suggests general involvement of the digestive system in this disease, although the presence of such involvement has not been fully elucidated. In addition, cases without systemic autoimmune diseases have been reported, which has led to the concept of an autoimmune related pancreatitis [2,3], so called "autoimmune pancreatitis" (AIP), proposed by Yoshida [2]. These findings support the hypothesis that an autoimmune mechanism may be involved in the pathogenesis and pathophysiology in a proportion of patients with pancreatitis [17-21], possibly mediated by an overactive apoptosis [7-10,22].

In the same respect, *Helicobacter pylori* infection has been strongly associated with peptic ulceration of the stomach [23] and gastric autoimmunity [24], and patients infected with *H. pylori* have been shown to possess autoantibodies that cross-react with antigens expressed on the gastric mucosa [24]. Moreover, as in the case of AIP, *H. pylori* is associated with autoimmune conditions like SjS (i.e., autoimmune sialadenitis), PBC, PSC, AIH or hepatitis C virus (HCV)-related liver disease which trigger autoimmune sequelae (AIH and HCV-related SjS) [25-35]. These *H. pylori*-related diseases are also characterized by fibrotic changes and/or lymphoplasmacytic inflammations [28,35-39], accompanied by aberrations of T cell apoptosis that contribute to hepatobiliary- or extrahepatic-tissue destruction [35,36,40-46]. Considering that *H. pylori* is involved in the pathogenesis and pathophysiology of the above mentioned autoimmune disorders [27-31,33], we propose that this organism might trigger AIP through induction of autoimmunity and apoptosis [47].

Definition and terminology of AIP

AIP can be defined as an inflammatory process of the pancreas due to an autoimmune mechanism establishing etiology of chronic pancreatitis [48]. Cases of isolated (primary) AIP without other autoimmune diseases have been reported. Many other terms, such as "primary inflammatory pancreatitis", "primary chronic pancreatitis", "non-alcoholic duct-destructive chronic pancreatitis", "lymphoplasmacytic sclerosing pancreatitis", "pseudotumorous pancreatitis", "granulomatous pancreatitis", "chronic inflammatory sclerosis of the pancreas", "pancreatitis showing the narrowing appearance of the pancreatic duct", and "sclerosing pancreatocholangitis" have also been used in the literature to identify AIP [4-6,49,50]. In addition, some authors defined "autoimmune exocrinopathy", "dry gland syndrome", or "a complex syndrome", as the concurrent involvement of the pancreas, the salivary glands (SjS) and the liver (PBC) [5,49]. However, it is poorly understood whether the pathogenetic mechanism of syndromic (or secondary) AIP with other autoimmune diseases is different from primary AIP. It was thought that there is a possibility of developing systemic autoimmune diseases in patients previously diagnosed as having primary AIP [3,48].

Strictly speaking, the classification of a disease as autoimmune requires a number of clinical/biochemical and experimental criteria not applicable to

human diseases [51]. A number of criteria have been suggested as indicative for an autoimmune pathogenesis [51]: 1) the presence of autoimmune antibodies specific for the disease and/or the presence of non-specific autoantibodies; 2) association with other autoimmune diseases; 3) association with HLA haplotype; 4) lymphocytic infiltration in the site of the disease, where HLA type II antigens are expressed; 5) responsiveness to steroid therapy. In this regard, AIP is associated with the coexistence of other autoimmune diseases, hypergammaglobulinemia (IgG4), presence of several autoantibodies (Table 1), histologic evidence mainly of lymphocyte infiltration and HLA-DR expression linked with T-lymphocyte mediated apoptosis, and a favorable response to steroid therapy [3,4,6], which represent clinical evidence of autoimmunity [52]. Notably, infectious agents are considered as causative agents and contributors to lesion expression in autoimmune disease [53].

Pathophysiology / pathogenesis of AIP (Fig. 1)

Occasional coexistence of pancreatitis with other autoimmune diseases [4-6,11] suggests that there may be common target antigens in the pancreas and other exocrine organs, such as the salivary glands, gastrointestinal or biliary tract, and renal tubules [3,4]. Several autoantibodies such as anticarbonic anhydrase II antibody (ACA-II) or antilactoferrin antibody (ALF) were frequently detected in patients with AIP, although these antibodies are not necessarily specific for AIP [3,4,6] (Table 1). Carbonic anhydrases (CA) are a family of zinc metal enzymes that catalyze the reversible hydratation of carbon dioxide to bicarbonate and hydrogen ions [49]. The enzymes are mainly distributed in the gastrointestinal tract, particularly in the salivary glands, stomach, duodenum, colon and biliary tract [4,49]. CA type II antigens are located in the pancreatic ductal epithelium. Thus, the presence of antibodies against this isoenzyme may provide evidence of an immune reaction to a pancreatic target antigen [3,4,49]. Similarly, lactoferrin (LF), a nonenzymatic protein, is also detected in various human tissues, including the lactating breast, bronchial, salivary, gastric glands or the pancreatic acinus [3,4], and the high prevalence of ALF in AIP suggests that LF may also be a candidate for the target antigen eliciting humoral and cellular-mediated immune responses in AIP [3,4].

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 [6]. Experimental evidence indicates that neonatally thymectomized (NTx) BALB/c mice, subcutaneously immunized with CA II or LF, and synergetic nude mice, with splenocytes transferred from disease induced NTx-mice, developed pancreatitis early, as well as sialoadenitis and cholangitis, while the normal BALB/c mice did not [3,50]. In immunized NTx mice, the prevalence of inflammation was significantly higher in the pancreas [54]. The effector cells were found to be T lymphocytes, especially Th1 type CD4+ T lymphocytes, mostly involved in the development of pancreatitis, sialoadenitis, and cholangitis [3,50]. CA II or LF immunized mice had apoptotic duct cells or acinar cells, respectively [54]. Expression of the interferon (IFN)-γ gene was upregulated in each group [54]. Similar findings were observed in the salivary glands and liver [54]. Therefore, an immunologic mechanism against CA II or LF is involved in the pathogenesis of these pancreatitis models, in which the effector cells are mainly Th1 type CD4+ T cells [54] exhibiting apoptotic activities. These T cell-mediated responses are accompanied by mononuclear and polymorphonuclear cell infiltration in the pancreas in the first three weeks and a consequent fibrosis in the most advanced stages (6 weeks) with progressive acinar atrophy [49].

Activated CD4+ and CD8+ T cells bearing HLA-DR were increased in peripheral blood lymphocytes and the pancreas of AIP patients [3,4]. HLA-DR antigens are expressed on the pancreatic duct cells as well as on CD4+ T cells, suggesting an autoimmune mechanism involved in inflammation [3,4]. Patients with AIP have the particular HLA haplotype DRB1*0405-DQB1*0401 [50]. CD4+ T cells are subdivided into Th1 and Th2 cells based on the profiles of cytokine production. Th1 lymphocytes, which produce interleukin (IL)-2, IFN-γ and tumor necrosis factor (TNF)-α, mediate cellular immunity, macrophage activation, cytotoxicity, and stimulate B cell to produce opsonizing and complement fixing antibodies [3,4]. In contrast, Th2 lymphocytes, which produce IL-4, -5, -6 and -10, pro-

Table 1 Characteristics of autoimmune pancreatitis [3-6,49,50]

Demographic Indices

Prevalence: 4.6-6.0% of chronic pancreatitis
Mean age: 59.1-59.4 years
Gender: Male preponderance (male/female ratio: 2:1)

Symptoms-Clinical Signs

No symptoms or only mild symptoms, frequently without acute attacks of pancreatitis
Obstructive jaundice (70-80%)

Radiologic Imaging

Diffuse enlargement of the pancreas ("sausage-like") on ultrasonography, CT, or MRI images
Occasional tumor-like local swelling of the pancreas
Diffuse or segmental irregular narrowing of the main pancreatic duct on endoscopic retrograde cholangiopancreatography (ERCP) images
Rarely, pancreatic calcification or cysts

Laboratory findings

Elevated levels of serum or urinary pancreatic enzymes, and CA19-9
Abnormal pancreatic exocrine function
Hypergammaglobulinemia or increased levels of serum IgG (37-76%)
Presence of autoantibodies [antinuclear antibody (ANA), anti-carbonic anhydrase antibody (ACA II), antilactoferrin antibody (ALF), anti-α-fodrin antibody (AFA), rheumatoid factor (RF), antismooth muscle antibody (ASMA), antineutrophil cytoplasmic antibody (ANCA)] (10-100%)

Histological findings

Fibrotic changes with infiltration of lymphocytes, plasma cells, and occasionally neutrophilic and eosinophilic granulocytes in the pancreas tissue
Occasional associated diseases
Other autoimmune diseases, such as Sjögren's syndrome, primary biliary cirrhosis or sclerosing cholangitis (12-60%)
Diabetes mellitus (type 1 and mainly type 2) (42-76%)
Effective steroid therapy
Usually effective for both the pancreas and biliary tract
Sometimes effective for diabetes mellitus

mote humoral and allergic responses [3,4]. Th1 cytokines may be critical in the induction and/or maintenance of AIP whereas Th2 cytokines may be implicated in disease progression [4]. Some AIP patients associated with diabetes mellitus also show evidence of autoimmune diabetes (type 1) by the presence of autoantibodies against glutamic acid decarboxylase, insulin, or tyrosine phosphatase-like protein [50]. Cellular-mediated immune responses may be implicated in the pathophysiology of autoimmune diabetes induced by Th1 cell-mediated apoptosis of insulin-producing β-cells [22].

The reported clinical and animal experimental aspects might lead to the following proposed pathogenetic sequence in AIP [3]: 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. CD8+ T cells may also act as cytotoxic cells.

This cell-mediated cytotoxic mechanism, involved in the pathogenesis of AIP mostly *via* apoptosis, appears to be reinforced by the following pathological findings observed in patients with AIP: (a) An intense inflammatory cellular infiltration mainly localizes around the medium-sized and large interlobular pancreatic ducts, but also involves the other pancreatic structures (acini, vessels, and nerves) [3,6,50]. The inflammatory infiltration consists mainly of lymphocytes and plasma cells but also contains some macrophages and occasionally neutrophilic and eosinophilic granulocytes [4-6,50]. Immunocytochemical typing of the lymphocytes reveals that the majority of them are CD4+ and CD8+ T lymphocytes, with fewer B lymphocytes [3,6,50]; (b) An increased expression of the major antigen of histocompatibility type II (HLA-DR) antigens is observed on the epithelial cells of the pancreatic ducts, which normally do not express these antigens, as well as on CD4+ T cells [3,4,6]; (c) The infiltration may be primarily subepithelial, with the epithelium only rarely being infiltrated by lymphocytes [50]. It completely encompasses the ducts and may narrow their lumen by infolding of the epithelium, often giving the lumen a starlike structure [50]. The periductal and ductal inflammation causes narrowing, obstruction and sometimes destruction of ducts. Extension of the inflammatory process to the acinar tissue leads to its replacement by fibrosis caused by such processes as necrosis/apoptosis [10], and, finally, the lobular architecture of the pancreas is almost lost [50]; (d) In a proportion of cases the chronic changes in the pancreas are overlain by "granulocytic-epithelial" lesions of the ducts [50]. This acute inflammatory component of AIP is characterized by focal detachment, disruption, and destruction of the duct epithelium due to invading neutrophilic and occasionally also eosinophilic granulocytes, which may also cluster immediately beneath the duct epithelium [50]. Occasionally, the granulocytic infiltration extends into the small intralobular ducts and acini [50]. These findings suggest that leukocyte recruitment and activation of the oxidative burst [55] may contribute to the pathophysiology of AIP; (e) T cells are involved in inducing apoptosis of acinar cells [7,56]. In particular, CD4 T cells exert direct cytotoxic effects through Fas ligand (FasL) expression [3], and FasL, TNF-α and TUNEL(+) apoptotic cells have been detected among pancreas-infiltrating cells [9]. These remarks indicate that apoptosis may be a key phenomenon in AIP.

Apoptosis and autoimmunity

Apoptosis, often synonymously used with the term 'programmed cell death', is considered a physiological form of cell death that involves the active participation of the dying cell in its demise [24,45,57]. Although apoptosis is equally important both for the development and for the maintenance of homeostasis in some adult tissues, suppression, overexpression or mutation of a number of genes which orchestrate the apoptotic process can also be associated with disease processes [45,57,58]. Focusing on the immune system, programmed cell death is required to destroy cells that represent a threat to the integrity of the organism [57,58]. Examples include: (a) cells infected with viruses. In this situation cytotoxic T lymphocytes (CTLs) execute virus-infected cells by inducing apoptosis [45]; (b) cells of the immune system. As cell-mediated immune responses decline, the effector cells must be removed to prevent them from attacking body constituents. Therefore, CTLs induce apoptosis in each other and even in themselves, thereby main-

taining the homeostasis of the immune system [57,58]. In this respect, programmed cell death plays a key role in regulating the size of the lymphocyte pool at several stages of lymphocyte maturation and activation [57,58]. Immature lymphocytes that do not express functional antigen receptors undergo programmed death. After their maturation, if lymphocytes never encounter antigens they die by apoptosis. Even if lymphocytes are activated by antigen, fractions of the progeny that do not receive sufficient growth factors or sustained stimulation also die [57,58]. During lymphocyte maturation and activation, fluctuations in the levels of expression of Bcl-2 (acronym for the B-cell lymphoma/leukemia-2 gene) or Bcl-x_L appear to correlate inversely with the susceptibility to apoptosis. Overexpression of Bcl-2 or Bcl-x_L leads to enhanced survival of immature lymphocytes and prolonged antibody responses [57,58]. Conversely, knockout of Bcl-2 or Bcl-x_L results in reduced survival of mature or immature lymphocytes. It has also been suggested that the long life span of memory lymphocytes may be due to constitutive expression of Bcl-2 and/or Bcl-x_L. Unlike Bcl-2 and Bcl-x_L, overexpression of Bax protein induces cell death upon growth factor withdrawal [58]. These proteins have been proposed to regulate the apoptotic procedure through both homo- and heterodimerization. Notably, T lymphocytes' death involves a series of proteases (caspases), which constitute the central executioners of apoptosis [58]. Therefore, physiological regulation of cell death is essential for the removal of potentially autoreactive lymphocytes. Defects in these apoptotic mechanisms are associated with autoimmune diseases such as rheumatoid arthritis, lupus erythematosus, inflammatory bowel disease or possibly AIP [11,58]. In fact, recent work has clearly demonstrated that dysregulation of apoptosis may underlie the pathogenesis of autoimmune diseases by allowing abnormal autoreactive lymphocytes to survive, and the inappropriate accumulations of activated T cells seem to be involved in the pathogenesis and perpetuate autoimmune disorders [58] including AIP [8,11]. In addition, infectious agents (bacteria/viruses) are considered as causative agents in the induction of autoimmune diseases [53,59]. In this regard, a strong association between AIP and gastric ulcer disease has been recently documented [11]. As *H. pylori* infection is strongly associated with peptic ulceration of the stomach [23], it is reasonable to propose that *H. pylori* may act as a trigger infectious agent that contributes to the pathophysiology of AIP.

Helicobacter pylori, autoimmunity and apoptosis

Helicobacter pylori is a gram-negative, spiral, flagellated bacterium with its large, circular chromosome comprising more than 1400 genes [23]. The bacterium is one of the most genetically diverse of all bacterial species that colonizes the gastric mucosa of most humans worldwide, mainly affecting older male adults in the developed world [60]. It has been estimated that more than one half of the world's population is infected with *H. pylori*. *Helicobacter pylori* infection is strongly associated with peptic ulceration of both the duodenum and the stomach [23]. Even though it has been early suggested that nearly all duodenal ulcers and most gastric ulcers were associated with *H. pylori* infection, more recent studies suggest that these early estimations were rather exaggerated. Nevertheless, perhaps 80% of the patients who have duodenal ulcers are infected with the organism, as are more than 60% of those with gastric ulcers [23].

In this respect, recent studies reported a high prevalence of gastric ulcer in patients with AIP [11], which also appears to affect mainly older male adults, thereby raising the possibility of an association between *H. pylori* infection and AIP [47]. This possibility is reinforced by a further association of both *H. pylori* infection and AIP with other autoimmune diseases including SjS, PBC, PSC or AIH. As in the case of AIP, these *H. pylori* related diseases are also characterized by fibrotic and/or lymphoplasmacytic inflammations [28,35-39] accompanied by aberrations of T cell apoptosis that contribute to hepatobiliary- or extrahepatic-tissue destruction [35,36,40-46]. Because *H. pylori* infection has now been implicated in the pathogenesis and pathophysiology of the above mentioned autoimmune disorders [27-31,33], this organism might also trigger AIP mainly through induction of autoimmunity and apoptosis [47].

Gastric autoimmunity is well established in patients with *H. pylori* infection associated with induction of autoantibodies that cross-react with the gastric mucosa [24,61]. The gastric H^+/K^+ ATPase located in canaliculi of parietal cells appears to be a target of this autoimmune response [61]. The presence of autoantibodies, in particular those directed to parietal cells, correlates with histological and clinical parameters of gastric mucosa atrophy. Therefore, *H. pylori* autoimmunity may play a critical role in the pathogenesis of chronic atrophic gastritis, a known risk factor for gastric ulceration or cancer [23,62]. It has been suggested, that molecular mimicry between *H. pylori* and the host on the level of Lewis x and y blood group antigens leads to the development of these autoantibodies [61]. *Helicobacter pylori* lipopolysaccharide (O-antigen region) expresses Lewis x and/or y blood group antigens in mimicry with human gastric epithelial cells. Mimicry may have two diverging roles in the pathogenesis of gastric mucosa injury. Infection may break tolerance and anti-Lewis antibodies may be induced to bind to gastric mucosa and cause damage probably by apoptosis. Secondly, mimicry may cause "invisibility" of the pathogen to the host, thus aiding persistence of infection [61]. It is relevant to note that *H. pylori* infection is associated with the synthesis of parietal cell autoantibodies, which, after eradication of the infection, persist and contribute to the recurrent antral chronic gastritis and intestinal metaplasia. Moreover, serum parietal cell autoantibodies are correlated with anti-*H. pylori* antibody titers [61]. Therefore, the serological titer of anti-*H. pylori* seems to reflect the autoimmunity status that correlates with gastric mucosal atrophy. This concept is further supported by the evidence indicating that the serological titer of anti-*H. pylori* IgG has been found to correlate with the histological grading of gastritis in patients with ulcer and non-ulcer dyspepsia. Thus, the titer of *H. pylori* serology may indirectly offer evidence of the severity of histological inflammatory changes [61].

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 [23,61,63], PBC [28,29] or possibly AIP, that induce apoptotic damage of neurons [23,61,63], liver tissue [40,42,43], or pancreatic tissue. Support for this theory is provided by reports showing that there is a positive association between the titer of anti-*H. pylori* antibodies and the titer of anti-pyruvate dehydrogenase antibodies in patients with PBC, and *H. pylori* infection could induce autoimmune responses in the development of both PBC and atrophic corpus gastritis [29]. Of note, apoptosis is a mechanism for cell surface expression of the autoantigen pyruvate dehydrogenase complex in patients with PBC [64]. Moreover, PBC patients positive for *H. pylori* have significantly higher values of alkaline phosphatase and prothrombin complex [28], indices reflecting liver tissue destruction. The most likely mechanism for the role of this organism is via molecular mimicry autoimmune sequelae. Future studies, however, are needed to support the hypothesis that the presence of IgG antibodies to *H. pylori* may adversely influence the pathophysiology of AIP [47] and other related autoimmune diseases.

Bacterial heat shock proteins (Hsps), particularly Hsp-60 or Hsp-70 of *H. pylori*, may represent major target antigens responsible for molecular mimicry causing autoreactivity between *H. pylori* and the host's immune gastric tissue. Due to the wide homology between bacterial Hsps and the mammalian counterparts, the humoral and/or cellular (T-cell) response against these proteins has been proposed to influence the pathogenesis of autoimmune diseases [23,61]. There is evidence that the presence of increased serum autoantibodies against Hsps may have pathogenetic importance by facilitating apoptotic cell death [61]. Because Hsps are recently being discussed as promising candidates for subunit vaccines, efforts to rule out the possibility or to demonstrate that *H. pylori* Hsps can trigger autoimmune mechanisms leading to autoimmune disorders such as SjS [23,61] or possibly AIP [47], and other autoimmune and vascular disorders must be considered.

Current studies indicate that apoptosis is a mechanism of cell death in several important *H. pylori*-associated upper gastrointestinal disorders and/or extraintestinal diseases, including autoimmune entities such as SjS, PBC, PSC, AIH [40-44] and possibly AIP [47]. *Helicobacter pylori* infection is associated with significant gastric epithelial cell damage including an increased level of apoptosis [61]. It also induces apoptosis of fibroblasts and smooth muscle cells in lamina propria. These alterations may be affected by exaggerated acid secretion, decreased

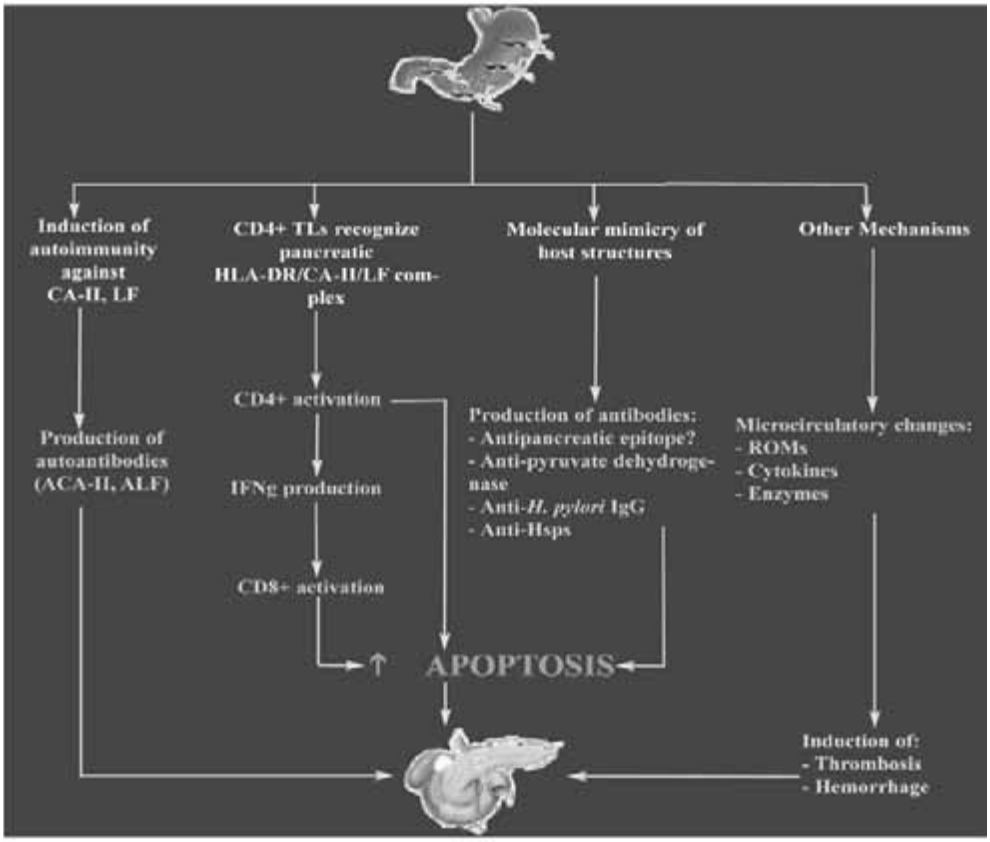

Fig. 1 Schematic presentation of the proposed pathophysiological mechanisms by which *Helicobacter pylori* infection might contribute to autoimmune pancreatitis (CA-II, carbonic anhydrase type II antigens; ACA-II, anticarbonic anhydrase II antibody; LF, lactoferrin; ALF, antilactoferrin antibody; TLs, T lymphocytes; IFN-γ, interferon-γ; anti-Hsps, antibodies against heat shock proteins; ROMs, reactive oxygen metabolites)

mucus protecting factors, and result in ulcer formation. In particular, *H. pylori* is found to change the expression of genes encoding growth factors, cytokine/chemokines and their receptors, apoptosis proteins, transcription factors, metalloproteinase-disintegrin proteins and tissue inhibitors of metalloproteinases that contribute to the pathogen-induced gastrointestinal and extradigestive disorders [61]. *Helicobacter pylori* infection leads to injury and cellular infiltration of T lymphocytes of the gastric epithelium by inducing activation of apoptotic surface markers on human lymphocytes and gastric epithelial cells and/or gastric adenocarcinoma cell lines. Moreover, *H. pylori* appears to upregulate the expression of HLA class II (HLA-DR) molecules on gastric epithelial cells and to induce their apoptosis probably through activation of T cells [61]. Gastric epithelium, in particular, may acquire antigen-presenting cell (APC) properties in *H. pylori* infection through *de novo*

expression of HLA-DR and co-stimulatory molecules. Macrophages in the lamina propria may also act as APC in the *H. pylori*-infected gastric mucosa. *Helicobacter pylori* possesses virulent factors promoting colonization (urease) that bind to class II major histocompatibility complex on gastric epithelial cells and induce their apoptosis [61]. On the other hand, elimination of *H. pylori* is associated with attenuation of HLA-DR expression on gastric epithelial cells and remission of mucosal inflammation. Taken together, these data support the interplay between *H. pylori* and epithelial cells in the course of *H. pylori*-mediated apoptotic gastropathy [61].

There is evidence suggesting that immune-mediated gastric epithelial cell apoptosis through Fas/FasL interactions participates in *H. pylori* disease pathogenesis [61]. Fas expression is abundantly increased on fundic gland epithelium, and FasL is detected on lamina propria mononuclear cells in *H. pylori*-infected mucosa, indicating that T cell-mediated cytotoxicity *via* Fas/FasL signaling may contribute to the induction of apoptosis in gastric epithelial cells during *H. pylori* infection. In particular, wild-type *H. pylori* strains increase Fas protein expression, and *H. pylori*-induced apoptosis involves the Fas/caspase cascade. Indeed, caspases -8, -3 and -7, are activated time-dependently by *H. pylori* as well as by the agonist anti-Fas [61]. Virulence factors possessed by *H. pylori* that induce tissue damage [lipopolysaccharide (LPS)] activate Fas/FasL-mediated caspase-8 release, and moreover stimulate cytochrome c release from the mitochondria, and subsequently activate caspases -9 and -3, leading to apoptosis, thereby suggesting that caspase-8 and mitochondria may play crucial roles in *H. pylori* LPS-induced apoptosis and that this accelerated apoptosis may be involved in abnormal cell turnover of *H. pylori*-infected gastric mucosa [61]. Additional studies also suggest that *H. pylori*-induced apoptosis in gastric epithelial cells is mediated by altered expression of the products of the Bcl-2 and Bax (increased expression of proapoptotic Bax and decreased expression of antiapoptotic Bcl-2) [61]. From another point of view, recent data suggest that the water-soluble surface proteins of *H. pylori* suppress neutrophil apoptosis. This may be caused by the suppression of FasL expression in neutrophils and Fas/FasL and TNF-Receptor1 expression on the surface of neutrophils. *Helicobacter pylori* water extracts also suppress the activation of caspases -8 and -3, and upregulate the expression of antiapoptotic Bcl-xL mRNA and proteins in neutrophils. The resulted prolongation of neutrophil life span could, in turn, contribute to the pathogenesis of *H. pylori* infection [55,61]. In addition, the longer survival of polymorphonuclear leukocytes (PMNL), induced by *H. pylori* LPS that suppresses spontaneous PMNL apoptosis, may also increase gastric epithelium injury in *H. pylori*-associated diseases, since longer survival and activation of PMNL provide a major source of reactive oxygen metabolites, which can cause tissue damage mainly in the absence of antioxidants [61].

As in the case of *H. pylori* infection, comparable T cell-mediated apoptotic signals and granulocyte recruitment and activation of the oxidative burst also contribute to the pathogenesis of AIP [3,4,7,9,10,50,56]. Therefore, in the perspective of the above-mentioned data, it is reasonable to suggest that, apart from the induction of autoimmunity, *H. pylori* might trigger AIP through a variety of apoptotic signals [47].

Finally, 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 [65]. Apart from reactive oxygen metabolites, active granulocytes and macrophages release proinflammatory cytokines (TNF, IL -1, -6 and -8), arachidonic acid metabolites (prostaglandins, platelet-activating factor and leukotrienes), proteolytic and lipolytic enzymes; these substances also interact with the pancreatic microcirculation to augment vascular permeability, which induces thrombosis and hemorrhage and leads to pancreatic necrosis [65]. *Helicobacter pylori* infection could exacerbate these events by promoting platelet and platelet-leukocyte aggregation, releasing large amounts of proinflammatory and vasoactive substances, such 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 [24,60,61,66].

In conclusion, we can consider that various autoimmune and apoptotic sequelae induced by *H. pylori* appear to influence the pathophysiology of

AIP, thereby suggesting an underlying link between *H. pylori* infection and AIP. If eradication of *H. pylori* infection may indirectly offer benefit to the 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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Review

New Concepts of Molecular Biology on Gastric Carcinogenesis

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SUMMARY
Gastric cancer not located in the cardia still remains the second most common cancer worldwide, whereas adenocarcinoma of the cardia and gastroesophageal junction has been rapidly rising over the past two decades. Gastric cancer can be subdivided into two distinct pathologic entities, diffuse and intestinal, that have different epidemiologic and prognostic features. Various genetic and environmental factors play important roles in gastric carcinogenesis; both lead to either abnormal genes overexpression or inappropriate expression of normal genes, whose products confer the malignant phenotype. Advances have been made in the genetic changes mostly of the intestinal type; its development is probably a multistep process, as has been well described in colon cancer pathogenesis, whereas it remains tentative whether the diffuse type of malignancy follows an analogous progression. The most common genetic abnormalities in gastric cancer tend to be loss of heterozygosity of tumor suppressor genes, particularly of p53 or "Adenomatous Polyposis Coli" gene. The latter leads to gastric oncogenesis through changes related to E-cadherin-catenin complex, which plays a critical role in the maintenance of normal tissue architecture. Mutation of any of its components results in loss of cell-cell adhesion, thereby contributing to neoplasia. E-cadherin/CDH1 gene germline mutations have been recognized in families with an inherited predisposition to gastric cancer of the diffuse type. Amplification and/or overexpression of putative trophic factors have also been observed in gastric cancer. Finally, *Helicobacter pylori* (*H. pylori*) infection is also involved in gastric carcinogenesis through various mechanisms, thereby necessitating *H. pylori* eradication in patients with gastric cancer.

KEY WORDS:
Gastric cancer; Epidemiology; Oncogenes; Tumor suppressor genes; Microsatellite instability; Apoptosis

ABBREVIATIONS:
Loss Of Heterozygosity (LOH); Inducible Nitric Oxide Synthase (iNOS); Vascular Endothelial Growth Factor (VEGF); Adenomatous Polyposis Coli (APC); Mutated in Colon Cancer (MCC); Familial Adenomatous Polyposis (FAP); Lymphoid Enhancer Factor (LEF); Cyclin-Dependent Kinase (CDK); Deleted Colon Cancer (DCC); Fragile Histidine Triad (FHIT); Nuclear Factor-κB (NF-κB); Cyclooxygenase (COX); Hepatocyte Growth Factor (HGF); Prostaglandins (PGs); HGF/Scatter Factor (HGF/SF); α-Fetoprotein (AFP); Interleukin (IL); Matrix Metalloproteinase (MMP); Chemokine IL-8 (CXCL8);

EPIDEMIOLOGY

Gastric cancer remains a significant worldwide health burden. Although the incidence and mortality rates of this malignancy not positioned in the cardia have been decreasing in the last decades, it still remains second only to lung cancer as a leading cause of cancer mortality worldwide (1). On the other hand, adenocarcinoma of the cardia and gastroesophageal junction appears to have increased in the past two decades in both hospitalized and population-based studies from several geographic regions. There is distinct geographical variation for gastric cancer, with the highest rates seen in the Far East. Japan ranks first worldwide in gastric cancer incidence, and fourth in gastric cancer mortality, trailing South Korea, Costa Rica and the former Soviet Union. Low incidence areas include Western Europe, North America, Africa and Australia (2).

In 1930, gastric cancer was the principal cause of cancer mortality in the United States for men and the third principal cause in women. Since that time, and following worldwide trends, the incidence of this disease has steadily declined. Importantly, the remarkable decrease of adenocarcinoma of the stomach in the United States during the last 70 years has occurred primarily for the intestinal type of the disease, which is associated with *Helicobacter pylori* (*H. pylori*) infection, achlorhydria and intestinal metaplasia. The incidence of the diffuse-type gastric cancer has remained constant over time. In contrast, apart from the proximal gastric cancers, there has been a relative increase in distal esophageal adenocarcinomas, particularly those associated with Barrett's esophagus (3). In 1996, it was estimated that 22,800 new cases of gastric cancer were diagnosed in the United States and that 14,000 people died of this disease; a fatal outcome shortly after this diagnosis is common. In 1997, gastric cancer was the eighth principal cause of cancer death in the United States, with the majority of patients being affected between the ages of 65-74 years, with the median age at diagnosis of 70 years in men and 74 years in women. Data collected worldwide demonstrate that a consistent predominance of gastric cancer in males (black and white) is seen worldwide, with about a 2:1 male:female ratio. In countries with a high incidence of gastric malignancy, the age at diagnosis tends to be a decade earlier. When gastric cancer affects younger patients, the male:female ratio is close to one, there is a high preponderance of blood group A, as well as a family history of cancer, and a higher inci-

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ABBREVIATIONS (Continued): Gastrointestinal Stromal Tumors (GISTs); Hereditary Nonpolyposis Colorectal Cancer (HNPCC); Mismatch Repair (MMR); Microsatellite Instability (MSI); Replication Error (RER)

dence of the diffuse type of gastric cancer than the intestinal type (2). Mass screening programs in high-risk regions such as Japan have helped in identifying and diagnosing gastric malignancies earlier, with some decrease in mortality rates, but significant improvements in tests for earlier diagnosis are needed.

Several studies suggest that rates of gastric cancer mortality increase with lower socioeconomic class. Most cases of the disease present at advanced stages and effective therapy is limited. A small but significant occurrence of cancer in gastric remnants has been reported in patients who have undergone gastrectomy for benign disease. A latency period of approximately 15-20 years has been noted before significantly increased risk.

These epidemiologic variables provoke intense efforts to identify new features in depth and strategies of molecular biology for better understanding the pathogenesis and/or management of gastric cancer.

TABLE 1 Oncogenes, Tumor Suppressor Genes and DNA Mismatch Repair Genes Involved in Gastrointestinal Tract Tumors

	Colon	Esophagus	Stomach
Oncogenes			
Ras	+		+
c-myc	+	+	+
c-erb B1			+
c-erb B2	+	+	+
hst-1	+		+
trk	+		
c-raf	+		
c-scr	+		
c-myb	+		+
c-yes			
c-mos			
c-fos			
Tumor Suppressor Genes			
p53	+	+	+
APC	+	+	+
DCC	+	+	+
MCC	+	+	+
DPC4	+		
DNA Mismatch Repair Genes			
hMSH2, hMLH1	+	+	+

TABLE 2 Genetic Changes in Gastric Adenocarcinoma

Changes	Gene	Frequency (%)
Suppression/Loss	p53	60-70
	FHIT	60
	APC	50
	DCC	50
	E-cadherin	<5
Amplification/Upregulation	COX-2	70
	HGF/SF	60
	VEGF	50
	c-Met	45
	AIB-1	40
	β-catenin	25
	K-sam	20
	ras	10-15
	c-erb B2	5-7
Microsatellite instability (MSI)		25-40
DNA aneuploidy		60-75

CLASSIFICATION - ETIOLOGY

According to Lauren's classification, gastric cancer can be subdivided into two distinct pathologic entities, diffuse (infiltrating or scattered malignant cells or islands of cells) and intestinal (gland-forming or expansive), that have different epidemiologic and prognostic features (4). The diffuse type of the disease involves two different subtypes: 'pure' (poorly differentiated carcinoma lacking any glandular structure) and 'mixed' type (coexistence of poorly differentiated carcinoma and intramucosal glandular structure). Diffuse gastric cancers exhibit signet-ring cells that aggressively infiltrate tissues, with nuclei that are eccentrically displayed by unsecreted mucus, and often presenting as linitis plastika. Apart from the same frequency throughout the world, diffuse-type gastric cancers present more diffusely in the stomach and earlier in life, arise without identifiable precursor lesions, tend to spread contiguously into the peritoneum, and are accompanied by a worse prognosis than the intestinal type. The intestinal type of cancers tend to predominate in high-risk geographic regions, tend to spread hematogenously, occur more distally in the stomach and later in life, arise in association with apparent precursor lesions (i.e. atrophic gastritis or intestinal metaplasia) and are characterized by the formation of gland-like tubular structures mimicking intestinal glands (3). Moreover, this type of malignancy is more closely linked to environmental and dietary risk factors and is the type of cancer that is now declining worldwide. The importance of distinguishing these two main histopathologic types of gastric cancer is highlighted by the finding of specific genetic changes associated with the different types.

It is now thought that the development of the intestinal type of gastric cancer is likely a multistep process, as has been well described in the pathogenesis of colon cancer. The progressive accumulation of genetic changes in both oncogenes and tumor-suppressor genes parallels the clinical and histopathologic progression from normal colonic epithelium through benign adenomas to frank colon cancer. This multistep nature of colon oncogenesis is directly illustrated by molecular experimental genetic studies, which demonstrate that the progression from adenoma to colon carcinoma results from the accumulation of molecular genetic alterations involving mainly 3 factors: activation of oncogenes; inactivation of tumor suppressor genes; and abnormalities in genes involved in DNA mismatch repair (5). The contention that the pathogenesis of the intestinal-type gastric cancer is also a multistep process, comprising gastric mucosal metaplasia-dysplasia-carcinoma sequence, is supported by the evidence that both atrophic gastritis and intestinal metaplasia are found in higher incidence in patients with intestinal-type cancer and in areas with high incidence of gastric cancer (4). This multistep

model of gastric cancer postulates that there is initially an inflammation, caused by *H. pylori* infection, as well as by exposure of toxins (preserved foods, high salt diet, bile salts), which can lead to the development of chronic active gastritis. In a subset of these patients, this inflammatory process leads to the development of atrophic gastritis, followed by intestinal metaplasia, dysplasia and, ultimately, early and advanced gastric cancer. It is considered that all stages prior to the development of high-grade dysplasia are potentially reversible, although this is still controversial. Unlike the case of colon cancer, the precise genes involved in each step of this progression are still not accurately defined. This is due to the fact that the premalignant stages of gastric cancer are not as readily identifiable endoscopically for prospective study compared with colon malignancy. In addition, several gastric tumors are very heterogeneous, containing a large proportion of normal stromal cells that may confound genetic analysis. Moreover, while there is increasing evidence that a genetic predisposition, in at least a subset of patients, plays an important role in gastric cancer, characterization of the timing of specific gene mutations in gastric cancer is made difficult at best. At present, it still remains tentative whether the diffuse type of gastric cancer follows an analogous histopathologic progression (2).

GENETIC FACTORS

The accumulation of multiple genetic alterations leading to oncogene overexpression, tumor suppressor loss and defective DNA mismatch repair is associated with tumors of the gastrointestinal tract, including gastric cancer (5,6) **(Table 1)**.

Gastric cancer, like all cancers, is considered to result, in part, from the accumulation of multiple genetic alterations leading to oncogene overexpression and tumor suppressor loss. In particular, gastric carcinomas are believed to evolve from native gastric mucosa or intestinal metaplastic mucosa that undergoes genetic and epigenetic alterations involving either the suppressor pathway (defects in tumor suppressor genes) or the mutator pathway (defects in DNA mismatch repair genes) (7).

A progress has been made in our understanding of the genetic changes that occur mostly in the intestinal-type gastric cancer. The most frequent genetic abnormalities found in gastric cancers tend to be loss of heterozygosity (LOH) of previously described tumor suppressor genes. Of note, mutations that disrupt the biological function of these genes have been found in association with cancers of the stomach, as well as esophagus and colon. The gene that has garnered the most attention is the tumor suppressor p53. 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 factors that allow DNA repair to occur or, if the damage is too great, factors which cause apoptosis (5). 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 ras oncogene in the pathogenesis of gastric cancer (8). 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. 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 (9). In this regard, p53 protein accumulation and increased expression of iNOS and VEGF might be responsible for gastric carcinogenesis and aggressiveness of gastric tumor (10). 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.

LOH at the 5q allelic locus, the site of the "Adenomatous Polyposis Coli" (APC) and "Mutated in Colon Cancer" (MCC) genes, occurs in over a third of gastric tumors but not in gastric dysplasia, with LOH being more common in the intestinal type regardless of stage (2). Focusing on APC, this gene resides on the long arm of chromosome 5. Inactivation of both copies of the APC gene has been found to be the "gate-keeping" event for the initiation of colorectal neoplasia. APC gene abnormalities may lead to disruption of normal cell-cell adhesion through altered association with molecules called catenins and cell adhesion molecule E-cadherin, which is a transmembrane glucoprotein that binds catenins. In particular, E-cadherin connects to the actin cytoskeleton through α- and β-catenin to establish cell polarity and mediates homophilic cellular interactions, indicating the involvement of E-cadherin in the formation of cell-junctions and the maintenance of epithelial integrity (11). Therefore, the E-cadherin-catenin complex is an important element for maintaining intercellular adhesion and plays a critical role in the maintenance of normal tissue architecture. Mutation of any of its components is believed to result in loss of cell-cell adhesion, thereby contributing to neoplasia, and is associated with poor differentiation and increased invasiveness of carcinomas (5). Mutations or losses of the APC

gene lead to susceptibility in colonic neoplasms in patients with Familial Adenomatous Polyposis (FAP), and somatic mutations of the APC gene occur in more than two thirds of sporadic colorectal carcinomas and adenomas including the smallest dysplastic lesions (5). Evidence supporting a role for APC in the pathogenesis of some forms of gastric cancer comes from the fact that FAP patients have a tenfold higher risk of developing gastric cancer compared with the general population. Mutations of APC gene occur in up to 20% of sporadic gastric cancers and gastric adenomas, mainly in well-differentiated intestinal gastric cancers in which up to 60% may have APC mutations (2). The mechanism of action of the APC gene is to sequester and inactivate cytoplasmic β-catenin preventing the formation of β-catenin/LEF (lymphoid enhancer factor), which acts as a growth-promoting transcription factor. It is known that β-catenin plays two distinct roles, in intercellular adhesion by E-cadherin already mentioned, and in transcriptional activation via TCF/LEF. Theoretically, the former role is tumor-suppressive, while the latter is oncogenic. β-Catenin mutations, preventing its inactivation by APC, are also found in an additional 16-27% of sporadic intestinal-type cancers. Important to note that, in intestinal-type gastric cancers β-catenin mRNA levels are greatly enhanced (12). High intranuclear levels of β-catenin protein play an important role in early tumor growth and may initiate the invasive processes in intestinal-type gastric carcinoma. In addition, β-catenin expression is lost in a subgroup of primary gastric cancers, is frequently absent in metastases and exhibits nuclear localization in cancers with either β-catenin or APC gene mutations. The loss of β-catenin expression in metastatic gastric cancers may result from hypermethylation of the β-catenin promoter. Therefore, the high frequency of either APC or β-catenin mutations, and/or LOH of the APC locus reported, suggest an important role of APC in the pathogenesis of gastric cancer (2,12). In this regard, the finding that LOH of APC is seen in gastric cancer but not in gastric dysplasia suggests that suppression of APC may be involved during this late stage of transition (2).

Genes that regulate entry into the cell cycle have also been involved in the pathogenesis of gastric cancer. The genes p16 and p27 inhibit entry into the cell cycle, and losses of p16 and p27 appear to play an important role during the gastric oncogenesis (13). Nearly half of gastric cancers are associated with significantly decreased expression of these genes. Absence of p27 [cyclin-dependent kinase (CDK) inhibitor] expression has been associated with a poor prognosis in gastric cancer (14). Absence of p16 expression correlates with poorly differentiated carcinoma but not with patient prognosis. The decreased expression of p16 and p27 occurs in the absence of detectable mutations and is thought to be secondary to hypermethylation. However, in the absence of clear-cut mutations, it is difficult to ascertain the overall significance of these genes (2).

A number of other genes have been reported to be either mutated or suppressed in gastric cancer, although their relative significance in the pathogenesis of gastric cancer remains to be determined (**Table 2**) (2,15).

These include "Deleted Colon Cancer" (DCC) gene, which is located on chromosome 18q, and its normal function is to promote proper cell-cell adhesion. Deletions and point mutations of DCC are present in sporadic colon cancer. DCC loss may lead to alterations in cell-cell interactions between colon cancer cells and facilitate metastasis (5). Specifically, LOH of DCC occurs in 35% and decreased expression occurs in 52% of gastric cancers, correlating with increased rates of liver metastases. Noteworthy is the fact that LOH of DCC occurs primarily in advanced intestinal gastric cancer and infrequently in early or advanced diffuse gastric cancer. A strong correlation between decreased DCC mRNA expression and level of gastric wall invasion, lymph node and liver metastases, or stage was found in cohesive (glandular+solid) and mixed tumors, but not in diffuse cancers (2,16). In this regard, the occurrence of both p53 and DCC genes mutations may cause gastric and colorectal cancers to become more malignant.

Fragile histidine triad (FHIT), a candidate tumor suppressor gene (17), and the fragile locus exhibiting susceptibility to carcinogen-induced alterations display LOH in 53% and decreased expression in 62% of gastric cancers. However, there are few data on the frequency of mutations of these genes in gastric malignancy, thereby also leaving their relative importance in the pathogenesis unclear (2).

On the other hand, oncogenes and the proteins encoded by them also appear to play a significant role in the pathogenesis of gastric cancer. These proteins constitute at least four distinct groups: a) peptide growth factors that may be secreted into the extracellular milieu; b) protein kinases, including receptor and nonreceptor tyrosine kinases and cytoplasmic serine/threonine kinases; c) signal transducing proteins associated with the inner cell membrane surface (membrane-associated G proteins that regulate generation of cyclic nucleotides); and d) nuclear transcriptional regulatory proteins [nuclear factor-κB (NF-κB)] (5).

The best studied and most common oncogene alteration in colonic neoplasm involves the ras oncogene, which also works together with p53 gene mutation in gastric carcinogenesis (8) and upregulates the gene expression of gastrin. The latter is an oncogenic growth factor contributing to gastric and colon carcinogenesis (5,17). Noteworthy, both low acid secretion and endogenous hypergastrinemia, especially in the elderly, may play an important role in differentiated and undifferentiated gastric carcinomas. Chronic hypergastrinemia in mice can synergize with *H. pylori* infection and contribute to eventual parietal cell loss and progression to gastric cancer (18). The gastric cultured epithelial cells exhibit the expression of gastrin receptors, and gastrin shows antiapoptotic activity through the upregulation of Bcl-2 and survivin. More-

over, gastrin stimulates the gene and protein expression of cyclooxygenase (COX)-2 and hepatocyte growth factor (HGF) in human cultured gastric cancer cells, thereby contributing to tumorigenesis (17). In this regard, *H. pylori* infection may contribute to gastric carcinogenesis via induction of gastrin and COX-2 that may account for the stimulation of tumor growth, angiogenesis and reduction in apoptosis (17). Therefore, *H. pylori*-positive patients developing gastric or colon cancer should be considered for *H. pylori* eradication to reduce the *H. pylori*-provoked hypergastrinemia and COX-2 overexpression in the tumor tissue.

COX is the key enzyme in the conversion of arachidonic acid to prostanoids. Two COX genes have been cloned, and expression of COX-2 mRNA and protein has been shown to be elevated in several human malignancies and in animal models of carcinogenesis. Moreover, recent evidence has implicated COX-2 in gastric, esophageal and colorectal carcinogenesis. Indeed, increased COX-2 expression was noticed in gastric carcinomas, Barrett's esophagus and esophageal adenocarcinomas, and colorectal adenomas and carcinomas (19). COX-2 appears to be mutagenic and tumorigenic *in vitro*. Moreover, COX-2 overexpression may inhibit apoptosis and increase invasiveness of malignant cells (19). In particular, COX-2 is expressed by the neoplastic cells in the intestinal-type gastric adenocarcinoma and by precarcinogenic (dysplastic) lesions leading to the development of gastric cancer, and its overexpression is associated with lymphatic metastasis, tumor invasion and differentiation of gastric carcinoma (20). CagA(+) *H. pylori* infection could upregulate the expression of COX-2 in gastric cancer in humans (20). Furthermore, *H. pylori* infection might activate NF-κB, an oxidant-sensitive transcription regulator of inducible expression of inflammatory genes such as COX-2, which regulates human gastric cancer cell growth and proliferation. Thus, oxidant-sensitive transcription factor NF-κB may play a novel role in the expression of COX-2 by *H. pylori* stimulation in gastric cancer cells (21).

Besides, COX-2 overexpression enhances prostaglandin (PG) synthesis and the importance of prostaglandins (PGE_2) in the progression of a chronic inflammation or neoplasia has long been recognized. Although the release of these compounds in response to tissue injury seems to be a key event in the reparative process and inflammatory response, it is becoming clear that they are implicated in cell proliferation and inhibition of immune surveillance; therefore, overproduction of PGs could favor malignant growth (19). Specifically, synthetic machinery and receptors for PGE_2, prominently expressed by T lymphocytes in gastric mucosa at the boundary of normal mucosa with tumor cells, may play a central role in prostanoid-driven tumorigenesis of this tissue (22). In addition, binding of HGF to its receptor (c-Met) regulates gastric cancer progression and metastasis, upregulates the expression of COX-2 gene and increases PG synthesis in gastric mucosa cells (23). On the other hand, inhibition of COX-2 prevents growth of gastric cancer xenografts in nude mice, and aspirin use (which inhibits both COX-1 and COX-2) decreases the risk of development of gastric cancer (2). Therefore, applying COX-2 selective (or nonselective) inhibitors reduces inflammation, suppresses carcinogenesis in the gastrointestinal tract and could be an effective and promising way to prevent gastric cancer (20,24).

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, HGF/scatter factor (HGF/SF), is also overexpressed in 67% of gastric cancers (2). Amplification of the c-Met gene is associated with increased depth of tumor invasion, lymph node and liver metastases and decreased survival (2). *H. pylori* activates the c-Met, promoting gastric cancer (25). Moreover, a higher frequency of c-Met expression is observed in α-fetoprotein (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 explanation for the poorer prognosis of AFP-producing gastric cancers, because HGF and its receptor, c-Met, are known to induce mitosis and cell movement and to promote tumor progression (26,27).

Amplification and/or overexpression of putative trophic factors have also been observed in gastric cancer. VEGF is a known angiogenic factor that promotes neovascularization of tumors, generally increasing the risk of invasion and metastases. It is noteworthy that VEGF is overexpressed in up to 54% of gastric cancers and correlates with the depth of invasion, the staging of gastric carcinoma, an increased risk of lymph node and liver metastases, and with disease recurrence (2,28). In general, the activation of coagulation, angiogenesis and inflammatory cytokines are considered to be related with tumor growth and metastasis. More specifically, in gastric cancer the plasma levels of VEGF and interleukin (IL)-6 are markedly increased in patients with stage IV disease and, thus, they might be useful for identifying metastatic gastric cancer patients (28). IL-6 may play a role in the angiogenesis of gastric carcinoma via modulation of VEGF. Furthermore, IL-8 acts as an angiogenic factor for human gastric carcinomas, upregulates matrix metalloproteinase (MMP)-9 expression and increases invasive activity of gastric carcinoma cells. Indeed, the IL-8 level in the neoplasms correlates significantly with the depth of invasion, venous invasion and lymphatic invasion. The chemokine IL-8 (CXCL8) appears to exert potent angiogenic properties on endothelial cells through interaction with its cognate receptors CXCR1 and CXCR2 (29). Relevant studies have shown than IL-8 directly enhances endothelial cell proliferation, survival and MMP expression in CXCR1- and CXCR2-expressing endothelial cells and regulates angiogenesis (18). IL-8 and VEGF may be independent and important prognostic factors in human gastric carcinomas (30). Moreover, the expressions of iNOS and VEGF are closely related to tumor angiogenesis and

are involved in the advancement and the lymph node metastases. Angiogenesis associated with VEGF may also play an important role in the progression of gastrointestinal stromal tumors (GISTs), and VEGF expression may serve as an indicator of poor prognosis (31). It is important to note that the association between high blood levels of VEGF and poor prognosis in cancer does not depend only on VEGF-induced stimulation of the neovascularization, but also on VEGF-related immunosuppression (VEGF inhibits dendritic cell maturation and lowers the antitumor cytokine IL-12). Thus, it would appear that VEGF, with or without the combination of IL-6, IL-12 and iNOS levels, may play a role in the development of advanced gastric cancer, and therapy with VEGF antibodies may be a potent therapeutic strategy against human gastric cancer (32).

Another main molecular pathway for carcinogenesis of the colon that appears to predominate in patients with hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) involves mutations in genes (hMSH2, hMLS1, hPMS1, hPMS2) that control the DNA mismatch repair process (5). DNA errors, called mismatches, often occur during DNA replication. During the synthesis of a new strand of DNA, DNA polymerase may create single-base pair mismatches or loop-outs of unpaired bases that tend to occur at repetitive DNA sequences termed microsatellites. These errors are normally repaired by enzymatic systems coded by "mismatch repair" (MMR) genes. If MMR genes are mutated, mismatch errors may affect genes important to cancer progression such as tumor suppressor genes, resulting in the development of malignancy (5). HNPCC arising tumors are characterized by a high level of genomic instability usually observed as alterations in microsatellite sequences that reflect a malfunction in DNA repair. This has been termed "microsatellite instability" (MSI) or the "replication error" (RER) phenotype. MSI has been observed in colorectal cancer from approximately 85% of HNPCC tumors compared with 15-26% of sporadic colon cancers (5). Specifically, HNPCC patients have an 11% incidence of gastric cancer, suggesting that MSI may also play a role in the development of gastric cancer (2). Low-level microsatellite (MSI-L) activity can be found in 40% of areas of intestinal metaplasia in patients with gastric cancer and in 14-20% of adenomatous polyps. MSI, caused most frequently by methylation of the hMLH1 promoter and found in 15-50% of sporadic gastric cancers, may have an important and early role in a subset of gastric cancers, particularly the intestinal type. The high-level MSI (MSI-H) subset of gastric cancer has features in common with its colorectal counterpart, exhibiting clinicopathologic profiles with good prognosis (33). Moreover, gastric tumors with MSI-H phenotypes have significantly lower COX-2 expression levels, which seem to favor better survival (34). However, contrary to Europe and the United States, advanced gastric cancers from Japan are more likely to demonstrate MSI, indicating prognostic differences in these MSI-related cancers (35). Summarizing, MSI may have an early role in gastric cancer pathogenesis and various prognostic significance.

The data regarding the genetics of diffuse gastric cancer are less complete. Mutations in the E-cadherin gene have been associated with the development of the diffuse type of gastric cancer (2,36). In particular, germline mutations in the E-cadherin/CDH1 gene have been recognized in families with an autosomal-dominant inherited predisposition to gastric cancer of the diffuse type (3,37). The criteria for familial gastric cancer are as follows: 1) there should be at least 3 relatives with gastric cancer; 2) one should be a first-degree relative of the other 2; 3) at least 2 successive generations should be affected; 4) at least 1 should be diagnosed before age 50; 5) other familial tumors should be excluded. Based on these criteria, of families with two or more cases of diffuse gastric cancer in first- or second-degree relatives younger than 50 years of age or three or more cases at any age, up to half may be attributed to inherited germline mutations in the E-cadherin/CDH1 gene (3). The cumulative lifetime risk of developing gastric malignancy in CDH1 mutation carriers is greater than 70%, and women of these families also have an increased risk for developing breast cancer. Due to this high risk of gastric cancer development, prophylactic gastrectomies have been performed in several unaffected CDH1 mutation carriers, and despite normal endoscopic evaluations and negative gastric biopsy specimens, pathologic foci of early gastric cancer were observed in all of the surgical specimens (3). However, prophylactic gastrectomy results in afflictions of life, and patients with a genetic risk for familial gastric cancer who reject this preventive total gastrectomy must be followed-up intensively by endoscopy and histology every 6-12 months (36). Therefore, a raised awareness among the physician community concerning this syndrome may allow for early detection and prevention of gastric and breast cancers in these high-risk individuals (3). Further evidence supporting the role for E-cadherin in gastric oncogenesis comes from studies showing that suppression of E-cadherin occurs in 51% of cancers, with a higher percentage found in the diffuse type of cancers. E-cadherin methylation is an early event in gastric carcinogenesis and is initiated by *H. pylori* infection (34). Furthermore, E-cadherin underexpression is associated with increased rate of lymph node metastases and decreased survival. Serum soluble E-cadherin is a potential valid prognostic marker for gastric cancer. A high concentration predicts palliative/conservative treatment and T4 invasion. The overall rates of E-cadherin mutations in gastric cancer are low, with the decreased expression of E-cadherin seen in gastric cancer likely secondary to hypermethylation of the E-cadherin promoter, which occurs in 50% of gastric cancers and 83% of diffuse gastric cancers. Expression of α-catenin is also decreased or absent in 68% of gastric cancers (2). In this respect, gastric adenocarcinoma in young patients has a poor prognosis, possesses aggressive histopathological features,

exhibits reduced expression of E-cadherin and β-catenin and demonstrates lower MSI than tumors in older patients (38).

Individuals with a family history of gastric cancer are more likely to develop atrophic gastritis (34% *vs.* 5%) in the setting of *H. pylori* infection. This genetic predisposition toward the development of atrophic gastritis may reflect different degrees of host immune response to infection. For example, IL-1 cluster polymorphisms have recently been identified as a risk factor for the development of atrophic gastritis and gastric cancer in *H. pylori*-infected patients but not in uninfected patients (2). Moreover, IL-1 gene cluster polymorphisms are associated with an increased risk of both hypochlorhydria, induced by *H. pylori*, and gastric cancer (39). The association with disease may be explained by the biological properties of IL-1-β, an important pro-inflammatory cytokine and a powerful inhibitor of gastric acid secretion. Polymorphisms in IL-1-β and its endogenous receptor antagonist are associated with risk of *H. pylori*-related gastric cancer (40). Furthermore, IL-1-β polymorphisms enhance not only IL-1-β but also IL-8 production in the gastric body and may play an important role in the development of atrophic gastritis. Also, IL-1-β polymorphisms are associated with increased risk of gastric cancer not only in whites, but also in patients from the Far East (Japan) (41). These last findings, as well as all previously mentioned data on the various mechanisms by which *H. pylori* is involved in gastric carcinogenesis, emphasize the need for *H. pylori* eradication in coping with gastric cancer.

Summarizing, in general, both genetic and environmental risk factors play a significant role in gastric carcinogenesis, leading to either abnormal genes overexpression or inappropriate expression of normal genes, whose products confer the malignant phenotype. Gene mutations could be either inherited (germline mutations) or acquired through various environmental risk factors or failure of intrinsic cellular mechanisms including DNA replication or transcription (somatic mutations). Specifically, the suppression/inactivation of several tumor suppressor genes and the activation of several growth-promoting genes appear to be important in the pathogenesis of gastric cancer. However, to date, there is no clear "gate-keeper gene" similar to APC in colorectal carcinoma, and the exact timing of the gene alterations in relation to the progression of gastric cancer remains to be elucidated.

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Review

Different Aspects in Functional Dyspepsia

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KEY WORDS:
Functional dyspepsia; Non-ulcer dyspepsia; *Helicobacter pylori* eradication; Visceral hypersensitivity; Gastric motility; Motor dysfunction; Psychological factors; Gastritis; Endoscopy; Histology; Placebo; Medications; Treatment; Management; Recommendations

ABBREVIATIONS:
Helicobacter pylori (*HP*); Functional Dyspepsia (FD); Non-Ulcer Dyspepsia (NUD); Gastroesophageal Reflux Disease (GERD); Irritable Bowel Syndrome (IBS); Number Needed to Treat (NNT); Gastrointestinal (GI); 5-Hydroxytryptamine (5-HT); Proton Pump Inhibitor (PPI); Psychological General Well-Being Index (PGWB); Intention-To-Treat analysis (ITT)

SUMMARY
Functional dyspepsia is still a puzzling medical problem. The causes are unknown, the pathogenetic mechanisms are uncertain, the management is controversial and medications are many times insufficient. The research so far has given conflicting results at all levels of investigation. This study represents an effort to collect all available data concerning the most disputed issues of functional dyspepsia. Topics regarding *Helicobacter pylori* eradication, pathophysiology, endoscopic and histologic correlations with symptomatology, the placebo effect and management options are presented following an evidence-based approach. Many articles, published in recent years, are discussed in order to obtain an overall insight of this peculiar symptom complex, named functional dyspepsia.

INTRODUCTION

Functional dyspepsia is a diagnosis of exclusion, defined as a persistent/recurrent pain or discomfort, of at least 3 months duration, located in the center of the upper abdomen while no structural or biochemical abnormality could be identified as the cause for the symptoms. Gastroesophageal reflux disease (GERD) and irritable bowel syndrome (IBS) must be excluded. Two major subgroups of patients, that of ulcer-like and dysmotility-like, have been proposed based on the predominant symptom. The predominant symptom is pain for the ulcer-like group and nausea, vomiting, early satiety, abdominal fullness or bloating for the dysmotility-like group (1).

Careful history, physical examination, upper endoscopy, basic hematological and biochemical tests and ultrasonography of the upper abdomen are the minimum requirements for the diagnostic evaluation of a dyspeptic patient.

A search of the PubMed database was performed focused mainly on randomized-controlled trials and articles written in the English language. Only in a few cases, articles not randomized, were used because of a lack of an adequate sum of evidence and published data.

HELICOBACTER PYLORI ASSUMPTIONS AND PROOFS

Much has been said about the role of *Helicobacter pylori* (*HP*) in functional dyspepsia. Several studies have been made to investigate if eradication of *HP* can have a beneficial result in the symptomatic relief of the patients. As for the randomized controlled trials with a respectful period of follow-up of at least 6 months that have been conducted in a more scrupulous manner the conclusions are diverse and sometimes conflicting (**Table 1**).

One such study published in 1996 included 41 patients that were treated with bismuth subcitrate for 1 month and metronidazole for 1 week or placebo and were followed up for 6 months. The intention-to-treat analysis showed a difference between the two groups with the eradication group achieving much lower symptom scores. There is one observation that is interesting and must be kept in mind. In 8 weeks the symptom score was considerably lower in both groups and at the same time the gastritis score was lower only in the eradication group but a difference in symptom relief was observed and increased in the following weeks till the end of the study period in favor of the eradication group (2).

Symptomatic, serologic and histological improvement at the end of the 1-year surveillance in 41 patients, randomly assigned to triple eradication therapy or placebo (H_2 blockers), was observed. The trial was published in 1996 and conducted in Taiwan (3). In a trial from a center in Ireland 100 non-ulcer dyspepsia (NUD) patients were randomized to receive bismuth-based triple therapy (metronidazole, tetracycline) or bismuth plus placebo for seven days and were followed up for 1 year. Patients that eradicated *HP* from their stomach in either group had a significant symptomatic improvement than those with the infection. In the placebo group 14% developed ulcers during follow-up (4). In the following year (1998) an interesting trial gave results in favor of *HP* eradication. 318 patients with NUD were randomized to take omeprazole plus antibiotics (amoxicillin-metronidazole) or omeprazole plus placebo for 15 days and then followed up for 1 year. Complete resolution of symptoms was observed in 21% in the first group and in 7% in the second group. One interesting part was that logistic regression analysis was used to examine for predictive factors of response to eradication treatment. The only factor that was considered significant was the duration of symptoms before treatment. Only 12% of the patients with more than five years duration of dyspepsia symptomatology had symptoms resolved in

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TABLE 1 Placebo Contolled Trials on the Eradication of *HP* in Functional Dyspepsia

Authors	Year of publ.	Country conducted	Total number of pat.	Treatm.	Placebo group A	Erad. group B	Duration rate in A	Outcome follow-up
Lazzaroni *et al.*	1996	Italy	157	CBS+metro x7d	Plac x7d	64%	6mo	Pos
Sheu BS *et al.*	1996	Taiwan	41	CBS x4w AM x2w	H2b x2mo	75%	1yr	Pos
Gilvarry J *et al.*	1997	Ireland	100	BMT x7d	B+Plac x7d	85%	1yr	Pos
Mc Coll K *et al.*	1998	Glasgow	318	OAM x15d	O+lac x15d	88%	1yr	Pos
Blum AL *et al.*	1998	Multinational multicenter	328	OAC x7d	O+Plac x7d	79%	1yr	Neg
Talley NJ *et al.*	1999	Multinational multicenter	275	OAC x7d	Plac x7d	85%	1yr	Neg
Greenberg PD *et al.*	1999	USA	100	OC x14d	Plac x14d	71%	1yr	Neg
Catalano F *et al.*	1999	Italy	126	BAM x7d	OA x7d	66.10%	2mo	Pos
Miwa H *et al.*	2000	Japan	90	OAC x7d	O+plac x7d	85%	3mo	Neg
Hsu PI *et al.*	2001	Taiwan	161	LAC x7d	Plac x7d	76%	2yr	Neg
Koskpato *et al.*	2001	Finland	136	OAM x2w+O x3mo	O+Plac x3mo	75%	1yr	Neg
Froehlich *et al.*	2001	Switzerland multicenter	147	LAC x7d	L+Plac x7d	52%	1yr	Neg
Malfertheiner P *et al.*	2003	Germany multicenter	800	LAC x7d	L x14d	65.60%	1yr	Pos
VeldhuyzenVZ *et al.*	2003	Canada multicenter	157	LAC x7d	Plac x7d	82%	1yr	Neg
Koelz HR *et al.*	2003	Switzerland	181	OA x15d	O x15d	52%	6mo	Neg
Kamada T *et al.*	2003	Japan	90	OAC x7d	Plac x7d	82.20%	3yr	Pos

Plac: placebo; d: days; w: week; mo: month; yr: year; pat: patients; CBS: colloidal bismuth subcitrate; H_2b: H2 blocker; OAM: Omeprazole + Amoxicillin + Metronidazole; LAC: Lansoprazole + Amoxicillin + Clarithromycin; OAC: Omeprazole + Amoxicillin + Clarithromycin; BMT: Bismuth + Metronidazole + Tetracycline; BAM: Bismuth + Amoxicillin + Metronidazole; pos: positive (statistically significant improvement of symptoms after eradication therapy); neg: negative (symptoms persisted after eradication therapy).

comparison to 27% of those with five years or less with dyspepsia in the group given eradication regimen (P=0.03). No other factor such as age, sex, smoking, predominant symptom or grade of acid secretion was predictive of treatment response. Another observation that merits some attention is the number of patients with ulcer formation during follow-up. There were four in the group given omeprazole and placebo and none in the group given eradication (5). This favors eradication of the infection as some patients with dyspepsia could develop ulcers in the future. The two previous studies were conducted in a single center thus making the possibility of a biased population sample with confounding factors greater and therefore not generalisable. Of course there were locally validated questionnaires that made the calculation of symptom scores more appropriate and objective (6).

A well-designed, multicenter study was the "OCAY" which was conducted in 1998 and was the first with a quality of life assessment. In this study there were strict inclusion criteria that left aside those that probably could have peptic ulcer or gastro-esophageal reflux disease. 328 patients were included in the intention-to-treat analysis and were randomized to receive either the classic triple therapy (omeprazole-amoxicillin-clarithromycin) or omeprazole and placebo antibiotics for one week. There was no statistical significant difference in symptom relief between the two groups at the end of the 12 months (27.4% *vs.* 20.7%) but there was a significant difference in gastritis healing (75% *vs.* 3%) in the intention-to-treat analysis. The study concluded that the eradication of *HP* is not likely to relieve symptoms. There are some interesting points to look at this study. The existence of up to five gastric erosions was not considered an abnormal finding in endoscopy and patients were included as having functional dyspepsia. Second even among the patients in the same group there was no difference in symptomatic improvement between those in whom *HP* was eradicated and gastritis healed and those that did not achieve either. The quality of life scores and gastrointestinal symptom scores were improved in both groups (two thirds) but without a statistically significant difference between them. The maximum rate of improvement was observed at the end of the first week of the trial and thereafter there was a lower rate of improvement until the 9th month when there was no further benefit or even worsening. In this study, the ulcer formation during follow-up also appeared more frequent in the placebo group [all ulcers (n=7) but one in the placebo group] (7). Another study (ORCHID) with similar characteristics to the previous one was conducted but this time the eradication group took the classic triple therapy and the control group took placebo for one week. Two hundred and seventy-five patients were included in the intention-to-treat analysis. The results showed no real benefit from eradication therapy. The symptomatic relief was not significantly different between the two groups (24% *vs.* 22%). At this study again there was no treat-

ment advantage between *HP*-positive and -negative patients but there was a difference when the patients were subdivided regardless of treatment in gastritis healed and non-healed (treatment successes 32% *vs.* 17%). Only 22% of those with healed gastritis were in the placebo group. It is remarkable that there was no association between the severity of symptoms and the grade of gastritis at baseline. In addition no difference was observed between the treatment and placebo group when they were subgrouped as ulcer-like and dysmotility-like patients (8).

In a meta-analysis of the two previous trials some benefit was observed after eradication treatment regarding the subgroup of ulcer-like dyspepsia for the reason that these patients have a diathesis for ulcer formation as time passes. In addition, it seems that by treating gastritis, dyspepsia symptoms were also improved because those patients with complete healing of gastritis had higher symptom success rate than those with incomplete healing (31% *vs.* 21% respectfully, $P=0.01$) (9). The OCAY and ORCHID trials come under criticism. The fact that these trials were multicenter and that only a few patients were included by each center makes these samples not representative of the population (less than 3 patients in 23 centers). On the other hand it seems that the questionnaires used for symptom validation were not validated in each country or center used. Nonetheless the results of a multicenter trial can more trustfully be generalized because local confounding factors in the population samples are more neutralized when the facts undergo statistical analysis (10).

Another randomized-controlled study conducted at the Department of Medicine of the University of California which included 100 patients with functional dyspepsia who were divided to take either omeprazole plus clarithromycin (500mg x3/24h) for 14 days or placebo and were prospectively followed up for 12 months. The astonishing finding in this trial was that patients with persistent *HP* infection showed more symptomatic relief than those with successful eradication even though this was not statistically significant (11).

In another study (1999) that looked at 126 elderly patients, a comparison was made between two treatment regimens, bismuth plus metronidazole and amoxicillin *versus* omeprazole plus amoxicillin, and the groups were followed up for only 2 months. The authors concluded that eradication of *HP* resulted in an improvement in functional dyspepsia symptoms in this patient category (12). The Frosch Study Group has checked on the results that antisecretory therapy has on functional dyspeptics in perspective to *HP* status. Seven hundred and ninety-two patients were randomized to receive for 14 days either placebo, ranitidine 150mg, omepruzole 10mg or omeprazole 20mg and were followed up for 6 months. In those patients who were *HP* positive there was a statistically significant difference in symptom relief between omeprazole 20mg and placebo. In *HP*-negative patients no such difference between placebo and any of the therapeutic regimens existed (13). In a study from Japan, 90 non-ulcer dyspeptics were included and randomized to receive for 1 week either a triple eradication scheme (omeprazole, amoxicillin, clarithromycin) or omeprazole and placebo. The follow-up lasted for 3 months. No significant improvement in symptoms was achieved in the treatment group compared to placebo although gastritis was significantly improved in the first group (14). A trial published in 2001 concluded that the eradication of *HP* does not benefit the symptomatology of functional dyspepsia patients. One hundred and sixty-one patients were assigned to either lansoprazole-triple therapy or placebo for 7 days. High symptom response rates were observed in both groups (treatment 58% *vs.* placebo 55%) that were not statistically significant. The same non-significant difference was found between the dyspepsia subgroups. A useful addition of this study was that the subsequent ulcer formation during a 1-year follow-up was determined. It was found that ulcer rates were significantly reduced in the ulcer-like subgroup compared to the dysmotility-like or unclassified subgroup thus it was concluded that eradication of *HP* could prevent subsequent formation of peptic ulcers in the ulcer-like patients with functional dyspepsia (15). Another randomized-controlled study was published in 2001, which divided 136 patients of functional dyspepsia into two groups. The first group received eradication therapy and the other placebo antibiotics. Both groups received omeprazole for 3 months and were followed for 1 year. There was no statistically significant reduction in dyspepsia symptom scores between the two groups (mean reduction 28.8% group A *vs.* 21.7% group B). No clear difference applied for the quality of life scores (16).

No relation to the duration of symptoms or the severity of gastritis and symptomatic relief after eradicating *HP* was observed in a randomized multicenter trial that was published in 2001. One hundred and forty-seven patients were divided into two groups to take either lansoprazole-based triple therapy or lansoprazole and placebo antibiotics for 7 days. The follow-up period was 1 year. There was no substantial benefit curing *HP* infection (17). The Frosch Study Group published in 2003 a double-blind randomized trial with a 6-month follow-up in order to investigate if eradication of *HP* in functional dyspepsia patients, who did not respond in previous acid-suppression therapy, could result in symptom resolution. One hundred and eighty-one *HP*-positive patients who did not respond to two weeks of antisecretory or placebo therapy were randomized to receive a two-week treatment either with omeprazole 40mg and amoxicillin 1g twice daily or omeprazole 20mg once daily. The symptom response after 6 months was 66% *vs.* 62% and was not statistically significant. This trial supported that those results were not in favor of eradication (18).

The ELAN study found that there is a symptomatic improvement after *HP* eradication. The first group took eradication regimen with lansoprazole and antibiotics (amoxicillin, clarithromycin) for 7 days and

was compared to the second group that took lansoprazole alone for 14 days. There was a treatment benefit in favor of the eradication group as much as 9% at the end of 1 year. According to the ITT analysis although dyspepsia symptoms improved in all groups early (at 6 weeks) after drug administration, this benefit was not sustained in the placebo group one year after but was still present in the group which took the eradication regimen (19). In a national multicenter trial from Canada including 157 functional dyspepsia (FD) patients, there was no treatment benefit between the group taking eradication regimen (lansoprazole + amoxicillin + clarithromycin) and the group taking placebo after one year of follow-up. Although there was an improvement in symptom score in both groups, in particular at the beginning of the trial (the first month), there was no statistically significant difference for those who took the active treatment (20). The above finding points to the strong placebo effect that confers either the treatment effort or the initial endoscopy.

A long-term follow-up (3 years) of 90 NUD patients with fundic atrophic gastritis and *HP* infection demonstrated a significant improvement of both symptoms and gastritis after eradication therapy. The patients were randomized to triple therapy (omeprazole + amoxicillin + clarithromycin) or placebo for 7 days (21). The patient selection having FD was based upon the criteria of AGA working party and not the ROME II definitions, meaning that patients with reflux-disease were included in the trial.

In spite of the patients' complaints in functional dyspepsia there are no predictive factors that could differentiate between *HP*-positive and -negative patients based on clinical presentation. Nonetheless overall symptom score and the degree of epigastric pain or weight loss are significantly more severe in *HP*-positive than -negative patients (22).

The presence of *HP* does not interfere with the natural history of functional dyspepsia. In a long follow-up of 201 functional dyspepsia patients lasting 6 to 7 years the effect of *HP* presence was evaluated. The results of this study revealed that the findings of several medical investigations during this period were similar in the two groups of *HP*-positive and -negative patients with the incidence of malignancy being zero and an annual incidence of ulcer disease of less than 1% (23).

In a placebo trial with a 5-year follow-up it has been demonstrated that there is a tendency of attenuation of symptoms over time independently of eradication of the infection. The severity of symptoms have no association with *HP* status at the end of the study period, nonetheless there was a significantly lower proportion of complete symptom response for those on placebo (24).

The serological status of *HP*-positive patients was evaluated in regard to symptom severity and histologic appearance of the stomach. 193 functional dyspeptics were divided into three groups, the first included *HP* negatives, the second *HP* positives and Cag-A positive and the other *HP* positives and Cag-A negative. No difference in symptom occurrence was observed and no more inflammation was noted between the groups (25). By contrast a cross sectional study published in 2001, included 422 patients with functional dyspepsia and concluded that mean symptom score was significantly higher in *HP*-positive-CagA-positive patients than *HP*-positive-CagA-negative or *HP*-negative dyspeptics (26).

It has been reported that high serum antibody titer against *HP* (IgG >50 units/mL) is an independent risk factor for frequent functional dyspepsia in dyspeptics and normal population. The *HP* infection and functional dyspepsia are related in at least this subset of *HP*-positive-high antibody titer-subjects (27).

The pretreatment gastric histology can be predictive of symptomatic response after eradication of *HP* in *HP*-positive functional dyspepsia patients. An acute inflammation score of more than three according to the updated Sydney system and an absence of lymphoid follicles were found to correlate with more symptom improvement after eradication with triple therapy (28). The higher the grade of active inflammation in the stomach the better is the eradication rate in *HP*-positive patients (29).

It seems that functional dyspepsia is more a symptom complex than a discrete disease entity. There is not a concrete pathophysiologic process discovered that could explain the diversity of appearing symptoms so far.

Epidemiological studies tried to prove a relation between *HP* and dyspepsia symptomatology. The prevalence of *HP* in the population is depending on age, socioeconomic status and ethnic background. Nonetheless studies have shown a greater prevalence of *HP* in dyspeptics than in normal subjects (48% vs. 36%) (30). The prevalence of *HP* infection and associated gastritis is about 30-50% among NUD patients. A relatively recent meta-analysis of epidemiological studies published in 2000 concluded that there is no evidence of a strong association between *HP* infection and dyspepsia but no one can exclude that a modest association could exist (31). Another epidemiological study (2263 patients) in Japan in 2001 found no association of *HP* status with the prevalence of symptoms that either imply ulcer-like or dysmotility-like dyspepsia (32).

Current recommendations of *HP* eradication in functional dyspepsia suggest that this is more an option than a strict obligation in the management of these patients since there is a subgroup that may benefit from such a therapy in the long term (33).

PATHOPHYSIOLOGY CONCEPTS

Pathophysiologic studies have not established a relation between *HP* presence and functional characteristics. It has been said that *HP* infected dyspeptics had no statistically significant difference in acid output, mucosal sensitivity to chemical or mechanical stimuli, peptic transit times or the migrating motor

complex duration compared to *HP*-negative dyspeptics or in many studies even to normal controls (30,34,35). It seems that the eradication of *HP* does not influence the gastric transit time for solids or the acid output but instead decreases the gastrin and pepsinogen I levels after 6 months of triple-based regimen that had been taken for 1 week (36). Gastric emptying of solids and liquids are not affected after one year of eradication of *HP* in a randomized controlled trial including 40 patients who were given either triple-based eradication regimen or placebo and 20mg omeprazole thereafter for another 3 months for both groups. The examination was scintigraphic (37). Gastric emptying was found to be delayed in 24% of 551 functional dyspeptics who were enrolled in a randomized controlled trial but this finding was associated neither with *HP* status nor severity of symptoms (38). The notion that atrophic gastritis provoked by *HP* delays gastric emptying and thus *HP* is a possible pathophysiological participant of FD, is presented in some trials. One recent study demonstrated long-term symptomatic benefit after eradication of *HP* in dyspeptic patients with fundic atrophic gastritis that was reversed after treatment (21).

Reduction of gastric inflammation three months after eradication of the infection was correlated with the ability of faster recovery of stomach shape after barostatic inflation of its proximal portion (39). Infection with *HP* has been related with decreased antral motility in manometric studies but in contrast scintigraphic studies of gastric motility have failed to prove a delayed gastric emptying in *HP*-positive patients. On the other hand it is not uncommon for motility dysfunction to be observed even in normal subjects with no dyspepsia symptoms or dyspeptics having constant abnormal motility tests either to present with sporadic symptoms or their symptoms to wax and wane. It seems that motility disorders operate through visceral hypersensitivity mechanisms which alter the perception of gastric distension but are under control of local or central neuronal systems that up or down-regulate the pain and sensation thresholds or that dysmotility and infection are two discrete mechanisms of action (40,41).

On the other hand it could be expected that the chronic inflammation caused by *HP* could alter local neuromuscular function by producing neurotransmitters such as somatostatin that has been proved to interfere in efferent and afferent neuronic signaling or by producing inflammatory mediators like cytokines (TNF, IL1, IL8) which in turn are known to provoke increased sensory perception (30).

The limited or no reduction in symptomatology of dyspepsia after eradication of the infection could be explained by the incomplete resolution of gastritis in many cases or by the production of proinflammatory cytokines by non-immune cells like the smooth muscle cells as experiments in animals have shown (34). A randomized-controlled trial including 271 FD patients looked into the histological changes induced by eradication therapy at 6 months after treatment with a triple-based regimen (pantoprazole, amoxicillin, clarithromycin). Active inflammation and *HP* infection was significantly decreased but chronic inflammation and intestinal metaplasia was slightly decreased and even persisted (42). It seems that many factors such as bacterial virulence, genetic susceptibility, host immune status and duration of disease may influence the symptom definition and response of any individual patient (34).

GASTRIC MOTILITY AND FUNCTIONAL DYSPEPSIA

Interdigestive and postprandial antral hypomotility has been reported in 50% of dyspeptics in manometric studies. Delayed gastric emptying of solids has been found in 30-60% of patients and delayed gastric emptying of liquids in 12-60% in scintigraphic studies (40). In a meta-analysis nearly 40% of patients with FD have been found with delayed gastric emptying. There was no association with symptoms of postprandial pain but they were associated with symptoms of gastroparesis. Nausea and other dyspeptic symptoms have been correlated with abnormalities of postprandial antral myoelectrical activity which when restored has been accompanied with symptom relief. In studies that have been restricted only to dysmotility-like dyspeptics a rapid emptying of intragastric contents has been observed from the proximal stomach accompanied by a prolonged distension and emptying of the antrum. Psychological factors like stress that seem to interfere with antral motility, gastric emptying and intragastric distribution of food are not always related with dyspeptic symptomatology or dyspeptics personality (40).

Gastric and intestinal hypersensitivity to distension that is quite specific for FD patients compared to other cases of organic dyspepsia has been observed in numerous studies. There is a prevalence of 30-40% among FD patients that present with hypersensitivity to gastric distension (43). Hypersensitivity has been related to abnormal gastric accommodation and is currently being demonstrated by invasive tests that use barostats to provoke gastric distention and to measure pain thresholds or sensation perception scores on a visual analogue scale. The lack of validated and standardized tests of evaluating visceral sensation has made difficult the interpretation of results. There seems to be many performance or interpretation errors in the tests' conduct and no clear proof that symptoms of FD could be attributable to abnormal sensations has been produced (44). Apart from the hypersensitivity reactions caused by mechanical disturbances it is also noted that altered gastric sensory perception can be due to the nutrient elements or caloric composition of the ingested food. This was observed during infusion of a high-fat meal in the duodenum, which caused exacerbated dyspeptic symptoms of satiety (43). More light has been shed into this questioning by a trial that included 160 patients and 80 normal controls in order to measure by barostatic evaluation the sensitivity thresholds to gastric disten-

tion and to correlate the results with dyspeptic symptoms. According to authors the most appropriate method for measuring hypersensitivity to gastric distension is the rate of increase of intraballoon pressure over intra-abdominal pressure in order to produce pain because it is independent of age and body mass index in contrast to absolute balloon pressure or balloon volume which are influenced by these factors. Sensitivity to gastric distension is found in 37% of the patients. Perception scores were higher among FD patients. Gastric emptying, gastric compliance and *HP* status were not different in patients with hypersensitivity in comparison with patients without. Lower perception and discomfort thresholds after balloon distention were associated with more intense symptoms of postprandial pain, belching and weight loss (45).

The autonomic nervous system and especially the vagal nerve has been implicated as a modulatory factor of sensorial perception. As the vagal nerve plays a role in neural gut reflexes causing proximal gastric relaxation and inhibition of antral motor activity it has been speculated that enhanced sensitivity of vagal afferents could result in altered gastric emptying and of course to symptoms of early satiety, nausea and fullness (41). Upon this latter concept it is also reported that vagal nerves are implicated in transporting signals of non-painful sensations such as nausea and early satiety and that receptors on afferent nerves either enhance (5-HT, CCK1) or reduce (leptin, GABA, k-opioid) that signaling activity (46).

A recent trial using normal controls in spite of the small number of dyspeptic patients enrolled (28 patients) have stated that autonomic dysfunction is significantly higher in FD than in controls by submitting the subjects to five standard cardiovascular autonomic reflex tests. A correlation could not be found among this autonomic dysfunction and visceral hypersensitivity or gastric emptying but a significant association between symptoms of epigastric pain and autonomic abnormalities was established (47). There are implications that pain in FD is a more generalized condition characterized by body hyperalgesia that is not confounded only to the GI tract (48).

Stress and anxiety are found to play a role in some pathophysiological processes that are considered as basic mechanisms of inducing dyspepsia. Psychological factors have been presented, in animal and human studies, to cause delayed gastric emptying, increased colonic transit times and visceral hypersensitivity. These stressors seem to act centrally altering the secretion of endogenous corticotropin-releasing factor (CRF) and inducing the secretion of serotonin peripherally in the tissues of the GI tract. By acting on their central and peripheral receptors respectfully, these substances affect gastric motility and sensitivity to various stimuli (49). More psychological disturbances (depression, anxiety) have been noted in FD patients (15.5%) in comparison either with normal population (7.1%, $P=0.01$) or other patients suffering from organic dyspepsia and organic diseases. This higher prevalence has been documented in an epidemiological study from China published in 2002 in which 1016 healthy individuals, 72 inpatients with FD and 281 inpatients with organic dyspepsia or other organic diseases, were interviewed (50).

Finally the notion that gastric acid is one of the causal factors for dyspepsia cannot be excluded. No gastric acid hypersecretion or increased acid-sensitivity has been observed in FD patients (51). On the other hand an increased exposure of duodenum to endogenous acid in FD patients associated with more severe dyspeptic symptoms has been reported. This duodenal acidification seems to provoke pathophysiological disturbances like inhibiting proximal gastric accommodation to a meal or increasing gastric chemosensitivity (52).

THE RELATION OF FD WITH ENDOSCOPIC AND HISTOLOGICAL FINDINGS: A FURTHER CONTROVERSY

A cross-sectional study from Norway that examined dyspeptic patients (309 patients) and normal controls (310 controls) found that 30-50% of mucosal inflammation was diagnosed in normal subjects and that 10% of both those who suffered from dyspeptic symptoms and those who did not had negative endoscopic examination. Only peptic ulcer and duodenitis were related with dyspeptic symptoms (53). Although histologically gastritis is more severe in *HP*-positive FD patients, clinical symptomatology is not correlated either with the degree of mucosal inflammation or the *HP* status (54). 27.5% of 167 NUD patients referred for investigation in a doctor's setting have normal endoscopy (55). Before eradication of the infection, mean significant association between symptoms and histological findings is higher than after 6 months of eradication treatment yet both symptoms and gastritis were decreased (56). Although significantly greater improvement in symptomatology is observed in *HP*-positive patients with antrum-predominant gastritis 1 year after eradication of the infection, overall (independent of gastritis type) no symptomatic benefit is greater between those with successful eradication and controls (57).

Erosive prepyloric changes were detected in 38.5% of 309 dyspeptic patients without significant association with any specific symptom or symptom complex of dyspepsia. Similar erosions were found also in 35.1% of 310 normal controls. No higher prevalence of *HP* infection was detected among patients with gastric erosive injuries. It seems that these endoscopic findings are not of clinical relevance in dyspepsia (58).

The correlation of discrete endoscopic/histologic findings and dyspeptic symptoms is confusing. The presence of gastritis and erosions is not considered relevant to the clinical presentation of dyspepsia by many investigators. Others believe that gross mucosal damage like ulcers and erosions must be considered as relevant causes of organic dyspepsia while superficial or atrophic gastritis could exist in patients diagnosed as having functional dyspepsia as they are not associated with any specific symptom pattern (59).

THE PLACEBO PROBLEM

Great symptomatic improvement ranging from 30% to 50% of patients taking placebo has been observed in many clinical trials (30,60). The placebo effect has been evaluated in a study including 30 patients with FD of moderate to severe intensity and long duration of symptoms (at least 2 years) that fulfilled a negative endoscopic examination before entering the trial. These patients took placebo for 2 months. The global and individual symptom score of a five-symptom complex was measured before and after the completion of therapy as well as the gastric motility and visceral hypersensitivity to distension. An 80% response rate was observed at 2 months of placebo treatment in all symptom scores constructed. There was no significant change in gastric motility or hypersensitivity to distension after placebo trial although a marked clinical symptom response had been measured. This placebo effect does not seem to interact with the suspected so far major pathophysiologic mechanisms (motility, sensitivity) but it most likely could act in a psychological or central level as a reassuring factor (60). Normal endoscopic results at the beginning of medical surveillance and the acceptance of a medication that is expected to help seems to release anxiety and take away the fear of a serious illness. Even the reputation of the physician that prescribes the recipe may act as a confidential and persuasive element of symptom relief. In the pathophysiological level the induction of secretion of endogenous opioid at the CNS caused by placebo may function as an analgesic factor. Placebo responses ranging from 6% to 69% were found in a meta-analysis, studying the effect of H_2-receptor antagonists and prokinetics in dyspepsia trials. These results were attributed to population differences, lack of validated outcome measures, different ways of defining a response and variability of dyspeptic symptoms over time (61).

The same characteristic reduction in symptom score soon after the termination of therapy, at the 1st or 8th week, independently of eradication of *HP* or gastritis grade, as demonstrated in many trials, is a point of strong suspicion for placebo-psychological effect.

WHAT TO USE

PPIs are demonstrating an antibactericidal and anti-inflammatory activity against *HP* infection and associated gastritis. The antibactericidal action is achieved by inhibition of bacterial urease or by regulation of the membrane of the organism. Inhibition of polymorphonuclear oxidative activity or NK T-cell cytotoxic effect by PPIs contributes to the decrease of host inflammation responses that precipitate gastritis. Lansoprazole and omeprazole are almost equally effective but pantoprazole is the least effective (62).

Independently of *HP* status the administration of 20mg of omeprazole for 1 month resulted in complete symptom resolution in 38% of FD patients compared to placebo ($P=0.002$). In dysmotility-like patients there has been no significant symptomatic improvement over placebo (63). The same results with a complete symptom relief in 31% and 15.5% on omeprazole 20mg bid and placebo respectively for 2 weeks was observed in another study (per protocol, $P=0.018$) (64). In a randomized-controlled trial involving only Chinese patients (n=453) the administration of lansoprazole for 1 month had no significant symptomatic benefit over placebo (65). In a meta-analysis H_2-receptor antagonists were found to exhibit a therapeutic gain of near 20% over placebo while cisapride showed a therapeutic gain of 36% in comparison with placebo although the latter conclusion seems to be in doubt because most trials had to do with highly selective patients in secondary centers demonstrating more of dysmotility-like syndromes (61).

Antidepressant medication (amitriptyline, clomipramine, desipramine, doxepin, mianserin) seems to be effective in relieving symptoms and in particular pain in FD with a NNT= 3.2 (66).

Fedotoxine is a K1-opioid receptor-agonist that acts in peripheral afferent nerves inhibiting the transmission of pain sensations to the brain. The use of this drug for 6 weeks reduces abdominal pain in FD patients as preliminary clinical studies have shown (67). Also 5-HT ligands play a modulatory motor and sensory role in the gut (68).

WHAT TO DO

The management strategy for handling dyspepsia consists of recommendations concerning both diagnosis and therapy.

Let's begin by saying that there is no clear evidence of benefit for eradicating *HP* in functional dyspepsia. The number of randomized studies, which are in support of eradication, is about the same with those that are against. Recent studies seem to be more sophisticated than previous ones and with longer follow-up periods but even this progress does not exclude methodological pitfalls as we still don't know the natural history and pathophysiology of this disorder very well. Risk factors for frequent and more severe manifestations of dyspepsia identified so far could be those of Cag-A seropositivity and high IgG antibody titers. Factors predictive of a better response to therapy are symptom duration of less than five years and high grade of antral-predominant active inflammation in the endoscopy and histology. Another study, in which the identification of factors that are prognostic of symptom response was not the primary outcome measure, found no correlation of histology or duration of symptoms with better treatment outcome. Higher symptom score, age between 36-45 years and "dyspeptic complaints at night" were found to be prognostic of better response (69). The fewer the days of dyspepsia in the first week of treatment administration with a proton pump inhibitor is a strong predictor of symptomatic response at 4 weeks. Age >40 years, duration of symptoms less than 3 months, low scores for epigastric pain (2-3 points of a 7 point Likert scale) and low impairment of vitality (>19 points of a 22 points in PGWB scale) were also prognostic factors of PPI treat-

ment response in FD patients taking omeprazole for 4 weeks (70).

For the time being it looks prudent to accept that eradication of *HP* may confer complete symptom resolution in the short term of about 10% over placebo and this results in a number needed to treat of 12 (69). Another reason for accepting eradication as a therapeutic option is that it gives protection to between 4% and 21% of NUD patients that will probably develop ulcer within 1 year if *HP* is still in the stomach (31). Eradication could be seen as an induction therapy for significant symptomatic improvement although symptoms do not seem to correlate with *HP* status in longer time periods.

For the patient that is *HP* positive there are two scenarios. Either test and treat for *HP* and if that fails one could proceed with PPI therapy or the other way around. The test and treat of *HP*, as the first action for those with no alarming symptoms or after failure of PPI administration, is an alternative and cost-effective strategy (71). Apart from the eradication of *HP* infection the use of PPI therapy as a single or double dose per day is considered beneficial in ulcer-like subgroup and prokinetics are the only available treatment for the dysmotility-like patients. In persistent cases a switch between PPIs and prokinetics is an option that could sometimes help. In refractory cases the use of psychotherapy and antidepressants or even hypnotherapy may be beneficial (71-73). The patient with dyspepsia, aged above 45 years, who has chronically taken NSAID or with alarming symptoms (anemia, blood loss, weight loss, dysphagia) must be immediately submitted to endoscopy. The real predictive efficacy of some of these factors for the presence of a serious illness has been recently questioned. They are found to be weak independent predictors (age, anemia, bleeding) of serious endoscopic findings with a sensitivity of 87% and a specificity of 26% (74). Follow-up endoscopy is recommended if dyspepsia symptoms recur in patients with risk factors for subsequent ulcer formation (advanced age, NSAID use, *HP* infection) (75).

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• *REVIEW* •

Role of *Helicobacter pylori* eradication in aspirin or non-steroidal anti-inflammatory drug users

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Abstract

Helicobacter pylori (*H pylori*) infection and the use of non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin at any dosage and formulation represent well-established risk factors for the development of uncomplicated and complicated peptic ulcer disease accounting for the majority of such cases. Although the interaction between *H pylori* and NSAID/aspirin use in the same individuals was questioned in some epidemiological studies, it has now become widely accepted that they are at least independent risk factors for peptic ulcer disease. According to data from randomized intervention trials, naive NSAID users certainly benefit from testing for *H pylori* infection and, if positive, *H pylori* eradication therapy prior to the initiation of NSAID. A similar strategy is also suggested for naive aspirin users, although the efficacy of such an approach has not been evaluated yet. Strong data also support that chronic aspirin users with a recent ulcer complication should be tested for *H pylori* infection and, if positive, receive *H pylori* eradication therapy after ulcer healing, while they appear to benefit from additional long-term therapy with a proton pump inhibitor (PPI). A similar approach is often recommended to chronic aspirin users at a high risk of ulcer complication. *H pylori* eradication alone does not efficiently protect chronic NSAID users with a recent ulcer complication or those at a high-risk, who certainly should be treated with long-term PPI therapy, but *H pylori* eradication may be additionally offered even in this setting. In contrast, testing for *H pylori* or PPI therapy is not recommended for chronic NSAID/aspirin users with no ulcer complications or those at a low risk of complications.

Key words: *Helicobacter pylori*; Aspirin

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INTRODUCTION

Salicylates have been used in therapeutic medicine since the Hippocrates' era and their use is still growing. During the last 50 years, there is a continuously increasing consumption of aspirin for cardioprotection and for secondary prophylaxis of recurrent stroke or other vascular occlusion, while the drug seems to have a possible role in chemoprevention of cancer and Alzheimer's disease[1-4]. Non-steroidal anti-inflammatory drugs (NSAIDs) are also widely used agents[5]. In the USA, it is estimated that more than 50% of the population over 65 years take aspirin or NSAIDs frequently[6].

The increasing widespread consumption of aspirin/NSAIDs, however, is associated with an increasing incidence of their well-known gastrointestinal complications, which include dyspepsia, gastric and/or duodenal erosions and ulcers and peptic ulcer complications. Peptic ulcer complications, usually bleeding, represent the most frequent serious adverse events of the use of aspirin/NSAIDs[7-9]. Peptic ulcer(s) may be found at endoscopy in up to 20-25% and ulcer complications requiring hospital admission develop in 2-5% of chronic users of NSAIDs[7-12]. Use of NSAIDs has also been shown to increase the risk of lower gastrointestinal bleeding[13]. The damaging effect of aspirin on the gastric mucosa may be less potent than the effect of NSAIDs[14]. Thus, it is estimated that the chronic use of aspirin increases the absolute annual risk of gastrointestinal bleeding by 0.04% (absolute annual risk of bleeding with and without aspirin: 0.09% and 0.05% respectively)[15]. Nevertheless, despite the relatively low absolute risk of bleeding in aspirin users, the numbers of aspirin related acute gastrointestinal bleeding episodes are rather high probably due to the huge numbers of individuals who take the drug regularly for long periods often having additional factors with increased risk for bleeding, such as old age and history of peptic ulcer disease. The use of selective NSAIDs, such as selective cyclo-oxygenase 2 (COX-2) inhibitors, significantly reduces but does not completely eliminate the risk of gastrointestinal complications[11,12,16], while their gastrointestinal benefit appears to be significantly restricted in cases of concomitant use of aspirin, even at low doses[11,17].

Helicobacter pylori (*H pylori*) is undoubtedly associated with the development of gastritis and uncomplicated and complicated peptic ulcer diseases[18]. Although the presence of two factors that can damage the gastric mucosa, such as *H pylori* and aspirin/NSAIDs, would be reasonably considered

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to increase the risk for development of uncomplicated and complicated peptic ulcer, data from several, mainly epidemiological, studies appear to be controversial and do not always confirm such an assumption[15]. This review focuses on the role of *H pylori* infection and the need for its eradication for prevention of gastrointestinal complications among aspirin/NSAIDs users by evaluating the relevant pathophysiological and epidemiological data as well as the results of the randomized, controlled clinical trials of therapeutic intervention.

PATHOPHYSIOLOGY

Aspirin or NSAIDs use is associated with the development of peptic erosions or ulcers through several mechanisms. First, aspirin acts locally through the release of salicylic acid in the stomach, which is not ionized by the gastric acid. Salicylic acid enters and accumulates within the gastric epithelial cells, is ionized intracellularly and disrupts cell metabolic functions increasing mucosal permeability and permitting the back diffusion of H^+ ions[16]. Moreover, aspirin promotes topical inflammation by inducing recruitment of leukocytes, which eventually results in capillary constriction and topical ischemia. The topical gastrotoxic effect of aspirin, however, does not seem to be particularly important, since it is associated only with superficial ulcerations that often resolve spontaneously despite the continued aspirin use[16]. The systemic gastrotoxic effect of aspirin is related to the inhibition of cyclo-oxygenase-1 (COX-1) and the subsequent disruption of prostaglandin synthesis and to the antiplatelet function that promotes bleeding complications[16]. The key role of the systemic effects of aspirin in the development of gastrointestinal complications is strongly supported by the data showing that the risk of such complications is independent of the drug formulation[17,21,22]. Even low doses of aspirin, such as 75 mg/d, have been shown to increase the risk of gastroduodenal ulcerations[21,22]. Inhibition of COX-1 with disruption of prostaglandin production is also the main mechanism of NSAIDs induced gastroduodenal complications[11,13].

H pylori infection induces a substantial inflammatory reaction in the gastric mucosa with recruitment of leukocytes and production of several inflammatory cytokines, which eventually result in attenuation of mucosal defense mechanisms[19]. Thus, *H pylori* infection and aspirin/NSAID use impaired gastric mucosal defense by different mechanisms and therefore an interaction between these two factors is biologically plausible.

EPIDEMIOLOGICAL STUDIES

The interaction between *H pylori* infection and aspirin/NSAIDs use in the development of ulcer and ulcer complications has been initially evaluated in several cohort or case-control studies. The findings of these studies, however, have been controversial, since some studies suggested an independent or additive role of *H pylori* infection and aspirin/NSAIDs use in gastrointestinal complications[23-35] and others proposed no association or even a protective role of *H pylori* infection in users of aspirin/NSAIDs[31-33]. Moreover, in one study, *H pylori* infection was found to increase the risk of gastric but not of duodenal ulceration in this setting[34]. The heterogeneity in study design and methodology, definitions, power, outcome, and selection of controls have been suggested to be responsible for such conflicting results[19].

In a systemic review published in 2002, the combined analysis of the data available up to October 2000 showed that there is synergism for the development of peptic ulcer and ulcer bleeding between *H pylori* infection and aspirin/NSAID use[34]. In particular, the presence of *H pylori* infection was found to increase 3-5-fold the risk of peptic ulcer in aspirin/NSAID users (prevalence of peptic ulcer in *H pylori* positive: 53% and *H pylori* negative: 21%, OR: 3.5) and 18-fold in subjects not taking aspirin/NSAID (prevalence of peptic ulcer in *H pylori* positive: 18% and *H pylori* negative: 0%, OR: 18.1)[34]. Thus, the risk of peptic ulcer is approximately 60-fold higher in *H pylori* positive aspirin/NSAID users compared with *H pylori* negative subjects not taking aspirin/NSAID[34]. Moreover, *H pylori* infection was shown to increase the risk of ulcer bleeding 1.8-fold, aspirin/NSAID use 4.85-fold, and the presence of both factors 6.1-fold compared with the risk of bleeding among *H pylori* negative subjects not taking aspirin/NSAID[34]. *H pylori* infection has also been found to increase the risk of upper gastrointestinal bleeding even in chronic users of low dose aspirin[27]. In a more recent case-control study from our group, *H pylori* infection was again found to increase the risk for upper gastrointestinal bleeding in aspirin/NSAID users 2.9-fold, or 1.7-fold when adjustment for other risk factors for bleeding was performed[28]. Taking all together, it seems that aspirin/NSAID use and presence of *H pylori* infection are at least independent risk factors for peptic ulcer and bleeding from peptic ulcer.

RANDOMIZED CLINICAL TRIALS

H pylori eradication in naive aspirin/NSAID users

If *H pylori* gastritis does enhance the risk for ulcer bleeding in aspirin/NSAID users, then *H pylori* eradication should substantially reduce such a risk in this setting. Since the risk of bleeding in aspirin/NSAID users is strongly related to the duration of drug use, being higher in subjects with new or recent drug onset (≤1-3 mo) than in chronic drug users (>1-3 mo)[36-38], the possible beneficial effect of *H pylori* eradication on naive aspirin/NSAID users was initially evaluated. In fact, only naive users of non-aspirin NSAIDs have been included in the relevant clinical trials to date, while the possible benefit of *H pylori* eradication in naive users of aspirin has not been evaluated yet.

H pylori eradication before NSAID use was found to significantly reduce the occurrence of peptic ulcers in 92 *H pylori* positive, NSAID naive patients with musculoskeletal pain treated with an 8-wk course of naproxen at a daily dose of 750 mg (peptic ulcers: 3/45 or 7% of patients in the *H pylori* eradication group *vs* 12/47 or 26% of patients in the placebo group, $P = 0.01$)[39]. In a longer trial with a similar design, *H pylori* eradication before NSAID use was again found to significantly reduce the risk of peptic ulcers in 100 *H pylori* positive, NSAID naive, patients with arthritis and a history of peptic ulcer or dyspepsia treated with a 6-mo course of diclofenac slow release at a daily dose of 100 mg (peptic ulcers: 5/51 or 12% *vs* 15/49 or 34%, $P = 0.0085$)[40]. In the latter trial, *H pylori* eradication

was also found to significantly reduce the risk of ulcer complications as well [6-mo probability: 4.2% (1.3-9.7) *vs* 27.1% (14.7-39.5), $P = 0.0026$][161].

H pylori eradication in chronic aspirin/NSAID users without a history of peptic ulcer complications

The results of the first large clinical trial of *H pylori* eradication in chronic NSAID users raised several questions for the benefit of such an intervention. In this trial[111], 285 *H pylori* positive chronic NSAID users with past or current peptic ulcers or NSAID-associated dyspepsia who continued a minimum dosage of NSAID for at least 6 mo were randomized to receive *H pylori* eradication therapy with omeprazole, amoxycillin and clarithromycin ($n = 142$) or omeprazole plus placebo antibiotics ($n = 143$) for 1 wk. Subsequently, all patients received omeprazole 20 mg daily for 3 wk followed by an additional 4-wk omeprazole course in cases with endoscopically detected peptic ulcers at 4th wk. The probability of being peptic ulcer free at 6th mo was similar in the *H pylori* eradication [0.56 (95%CI: 0.47-0.65)] and the omeprazole-control group [0.53 (95%CI: 0.44-0.62)], while healing of gastric ulcers was significantly impaired in the *H pylori* eradication group (gastric ulcers healed at 8th wk: 72% in the *H pylori* eradication group *vs* 100% in the omeprazole-control group, $P = 0.006$)[161].

The design of the latter trial, however, was different from the design of the trials in naive NSAID users, since *H pylori* eradication therapy was given to subjects with ulcers or at high-risk of ulcers, who had already been on long-term NSAID consumption. Moreover, both the *H pylori* eradication and control groups were treated with 4-8 wk of omeprazole for ulcer healing. The lower probability of gastric ulcer healing at 8th wk in the *H pylori* eradication group should be associated with the more potent antisecretory activity of the PPIs including omeprazole in the presence than absence of *H pylori* infection[162]. Similar findings have also been observed in another large study including 692 chronic NSAID users, in which gastric ulcer healing with ranitidine or lansoprazole was shown to be significantly enhanced in the presence of *H pylori* infection (healing of gastric ulcers at 8th wk: 70% in *H pylori* positive *vs* 61% in *H pylori* negative, $P<0.05$)[163]. According to these data, it has been reasonably suggested that any attempt to eradicate *H pylori* infection should follow ulcer healing in the management of chronic NSAID users, although the efficacy of such an approach remains to be tested. The efficacy of *H pylori* eradication in chronic aspirin users has not been evaluated yet.

H pylori eradication in chronic aspirin/NSAID users with a recent peptic ulcer complication

Subjects with a history of upper gastrointestinal bleeding or other peptic ulcer complications represent a particular subgroup of aspirin/NSAID users who are at a high risk for recurrent bleeding during continued aspirin/NSAID use[164,165]. Strategies that may prevent bleeding in this setting include concurrent therapy with a PPI or eradication of *H pylori* infection in *H pylori* positive subjects. The efficacy of these two strategies was evaluated in a large clinical trial including 400 *H pylori* positive aspirin/NSAID users with a history of upper gastrointestinal bleeding[166]. All patients initially discontinued aspirin or NSAID therapy and were treated with omeprazole 20 mg daily for at least 8 wk to promote ulcer healing. Once the healing of ulcer was confirmed, 250 patients who were given 80 mg of aspirin daily for heart disease or stroke and 150 patients who were given 500 mg of naproxen twice daily for arthritis, both for at least 6 mo, were separately randomized to receive 20 mg of omeprazole daily for 6 mo or a 7-d course of *H pylori* eradication therapy followed by placebo once daily for 6 mo. In patients taking aspirin, no significant difference in the probability of recurrent bleeding during the 6-mo follow-up period was observed between those who received *H pylori* eradication therapy (1.9%) and those who received omeprazole (0.9%) (absolute difference: 1%, 95%CI: -1.9-3.9%). In contrast, in patients taking naproxen, the 6-mo probability of recurrent bleeding was significantly lower in the omeprazole (4.4%) than in the *H pylori* eradication group (18.8%) (absolute difference: 14.4%, 95%CI: 4.4-24.4%, $P = 0.005$)[166]. According to these data, it seems that, after ulcer healing, *H pylori* eradication may be effective in preventing recurrence of upper gastrointestinal bleeding in chronic aspirin users, but not in chronic NSAID users, who require long-term potent antisecretory therapy with a PPI.

Whether the combination of *H pylori* eradication and long-term use of PPIs may further decrease the risk of recurrence of peptic ulcer complications in chronic aspirin users was evaluated in a recent clinical trial[167]. Thus, 123 *H pylori* positive patients with a history of an aspirin-related peptic ulcer complication and current peptic ulcer were initially treated with a 7-d *H pylori* eradication therapy followed by 40 mg of famotidine daily for 5 or 13 additional weeks until ulcer healing. Then, they all restarted taking 100 mg of aspirin daily and randomized to receive 30 mg of lansoprazole daily or placebo. During a median follow-up of 12 mo, recurrence of ulcer complications was observed in 9 (14.8%) of 61 patients in the placebo group and in only 1 (1.6%) of 62 patients in the lansoprazole group (adjusted hazard ratio: 9.6, 95%CI: 1.2-76.1, $P = 0.0088$)[167]. It should be noted, however, that four of the nine placebo-treated patients with a recurrence of ulcer complications were reinfected with *H pylori* and an additional two patients of this group took other NSAIDs. Despite these problems in the latter trial, it is becoming widely accepted that long-term therapy with a PPI after *H pylori* eradication offers additional benefit in preventing peptic ulcer complications in high risk *H pylori* positive chronic aspirin users[107].

DISCUSSION

All existing data suggest that the presence of *H pylori* infection represents an additional risk factor for peptic ulcer complications in aspirin/NSAID users[105,168]. However, in current clinical practice which should be guided by the evidence-based medicine and should take into account the cost/benefit analysis of any major intervention, the management of *H pylori* infection and generally the gastrointestinal prevention in aspirin/NSAID users should probably be individualized (Table 1). Thus, the optimal management of such subjects appears to depend on the main factors affecting the risk of ulcer complications, which are: (1) whether the subject is a

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Table 1 Recommendations[93-95] and evidence for the *H pylori* test-and-treat approach and/or the long-term therapy with a proton pump inhibitor (PPI) in users of aspirin or non-steroidal anti-inflammatory drugs (NSAIDs)

	H pylori test-and-treat approach	Long-term PPI therapy
Naive aspirin users	Recommendation	No
Naive NSAIDs users	Recommendation-evidence[96-98]	No
Chronic aspirin users		
With a recent ulcer complication	Recommendation[1]-evidence[96,97]	Recommendation-evidence[99]
At high risk for ulcer complication	Recommendation[2]	Recommendation
At low risk for ulcer complication	No	No
Chronic NSAIDs users		
With a recent ulcer complication	Potential benefit[1,2]	Recommendation-evidence[99]
At high risk for ulcer complication	Potential benefit[1,2]	Recommendation
At low risk for ulcer complication	No	No

[1]*H pylori* eradication therapy in chronic users of aspirin or NSAIDs with a recent ulcer complication or those at a high risk should be administered after confirmation of ulcer healing. [2]*H pylori* eradication therapy in chronic NSAIDs users with a recent ulcer complication or those at a high-risk may be given as a potentially beneficial intervention in addition to the long-term PPI therapy.

naive aspirin/NSAID user or already on long-term (>1-3 mo) or chronic drug use[96-98]; (2) whether the subject is at high risk for bleeding or other complication of peptic ulcer (history of complicated or uncomplicated peptic ulcer, age older than 60-65 years, recent dyspepsia, treatment with anticoagulants)[6,36-38,93]; and (3) perhaps whether they take aspirin or non-aspirin NSAID[95,98].

In naive NSAID users, it is well accepted and supported by strong data[95,98] that they should be tested for the presence of *H pylori* infection and, if positive, receive *H pylori* eradication therapy before NSAID use[95-98]. A similar strategy is also suggested for naive aspirin users[93], although the efficacy of such an approach has not been evaluated yet.

In chronic aspirin/NSAIDs users, the recommendations may depend on the risk for peptic ulcer complications[6] and the type of drug. The indication for use of aspirin or NSAIDs should be first evaluated in all such users at high risk for peptic ulcer complications. Moreover, the probability and the cost/benefit of replacement of aspirin or NSAID with a less gastrotoxic antiplatelet agent or a selective COX-2 inhibitor respectively may be considered[6,11,12].

All individuals, who should continue taking aspirin after development of a peptic ulcer complication, should be tested for the presence of *H pylori* infection and, if positive, receive *H pylori* eradication therapy after peptic ulcer healing. In addition, they should subsequently receive long-term therapy with a PPI[11,47]. A similar approach may be recommended in chronic aspirin users without a recent ulcer complication but at high risk for ulcer complication, such as those with a history of peptic ulcer[93]. It should be noted, however, that there are no strong data to support the combined prophylactic approach with both *H pylori* eradication and long-term PPI therapy in this setting.

All individuals, who should continue taking NSAIDs being at high-risk for peptic ulcer complication, certainly benefit from long-term therapy with a PPI[93,80,99]. The risk of relapse of ulcer complication in chronic NSAIDs users taking PPI, however, is higher than the risk of such a relapse in aspirin users irrespective of the type of gastroprotection[100]. Thus, given that *H pylori* infection represents an independent risk factor for gastrointestinal bleeding in chronic NSAIDs users[101], it is often recommended that testing for *H pylori* infection and, if positive, *H pylori* eradication therapy should be offered to high-risk chronic NSAIDs users in addition to the long-term PPI therapy[93], despite that there are no strong data to support such an approach.

Finally, testing for *H pylori* infection or PPI therapy is not recommended for chronic users of aspirin or NSAIDs with no peptic ulcer or complication or those at a low risk of the same[93].

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Autoimmune Pancreatitis, *Helicobacter pylori* Infection, and Apoptosis: A Proposed Relationship

To the Editor:

Autoimmune pancreatitis (AIP), an inflammatory process of the pancreas due to an autoimmune mechanism establishing the etiology of chronic pancreatitis, is characterized by a male adult preponderance, the presence of autoantibodies, hypergammaglobulinemia, pancreatic enlargement, pancreatic duct strictures, and pathologic features of fibrotic changes with intense, mainly lymphocytic infiltrations, which may contribute to tissue destruction, probably by apoptosis.[1,2] In almost 60% of the cases, this type of pancreatitis coexists with other autoimmune diseases such as Sjögren syndrome, sclerosing extrahepatic cholangitis, primary biliary cirrhosis (PBC), autoimmune hepatitis, or other extrapancreatic disorders, and recently with gastric peptic ulceration.[2,3] The diversity of extrapancreatic lesions with similar histopathologic findings suggests general involvement of the digestive system in this disease, although the presence of such involvement has not been fully elucidated. Similar to AIP, *Helicobacter pylori* infection, a well-known cause of gastric ulcer, which also appears to affect mainly older male adults, has been associated with the same autoimmune conditions, via molecular mimicry of host structures by its constituents.[4] 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. Considering that *H. pylori* is involved in the pathogenesis and pathophysiology of these autoimmune disorders, we propose that this organism might trigger autoimmune pancreatitis through induction of autoimmunity and apoptosis.

Occasional coexistence of pancreatitis with other autoimmune diseases[2,3] suggests that there may be common target antigens in the pancreas and other exocrine organs, such as the gastrointestinal tract.[2] Several autoantibodies such as anti-carbonic anhydrase II antibody (ACA-II) and anti-lactoferrin antibody (ALF) were frequently detected in patients with AIP.[2] Carbonic anhydrases (CA) are chiefly distributed in the gastrointestinal tract, and CA type II antigens are located in the pancreatic ductal epithelium. Thus, the presence of antibodies against this isoenzyme may provide evidence of an immune reaction to a pancreatic target antigen.[2] Similarly, lactoferrin (LF), a nonenzymatic protein, is also detected in various human tissues, including the gastric glands and the pancreatic acinus,[2] and the high prevalence of ALF in AIP suggests that LF may also be a candidate for the target antigen eliciting humoral and cellular-mediated immune responses in AIP.[2]

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. In this respect, activated $CD4^+$ and $CD8^+$ T cells bearing HLA-DR were found to be increased in peripheral blood lymphocytes and the pancreas of AIP patients.[3] HLA-DR antigens are expressed on pancreatic duct cells as well as on $CD4^+$ T cells, suggesting that an autoimmune mechanism is involved in inflammation.[3] The reported clinical and animal experimental aspects might lead to the following proposed pathogenetic sequence in AIP: 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, $CD4^+$ or $CD8^+$ 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, as indicated by a number of pathologic findings observed in patients with AIP.

Recent work has clearly demonstrated that dysregulation of apoptosis may underlie the pathogenesis of autoimmune diseases by allowing abnormal autoreactive lymphocytes to survive, and the inappropriate accumulations of activated T cells seem to be involved in the pathogenesis and perpetuate the autoimmune disorders[5] including AIP.[1] In addition, infectious agents (bacteria/viruses) are considered causative agents in the induction of the autoimmune disease.[6] In this respect, a strong association between AIP and gastric ulcer disease has been recently documented,[3] and, as *H. pylori* infection is strongly associated with peptic ulceration of the stomach, it is reasonable to speculate that *H. pylori* may act as a trigger infectious agent that contributes to the pathophysiology of AIP.

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,[7] PBC, or possibly AIP that induce apoptotic damage of neurons, liver tissue, or pancreatic tissue. Support for this theory is provided by reports showing that there is a positive association between the titer of anti–*H. pylori* antibodies and the titer of anti-pyruvate dehydrogenase antibodies in patients with PBC, and *H. pylori* infection could induce autoimmune responses in the development of both PBC and atrophic corpus gastritis. Moreover, PBC patients positive for *H. pylori* have significantly higher values of alkaline phosphatase and prothrombin complex, indices reflecting liver tissue destruction. The most likely mechanism for the role of this organism is via molecular mimicry autoimmune sequelae. Future studies, however, are needed to support the hypothesis that the presence of IgG antibodies to *H. pylori* (the titer of which

indirectly offers evidence of the severity of histologic inflammatory changes) may adversely influence the pathophysiology of AIP and other related autoimmune diseases.

Bacterial heat shock proteins (Hsps), particularly Hsp-60 and Hsp-70, of *H. pylori* possess a wide homology with mammalian counterparts; thus, the humoral and/or cellular (T-cell) responses against these proteins have also been proposed to influence the pathogenesis of autoimmune diseases, possibly including AIP. In particular, autoantibodies against Hsps may have pathogenetic importance by facilitating apoptotic cell death.

Finally, 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. Active granulocytes and macrophages release reactive oxygen metabolites, proinflammatory cytokines (tumor necrosis factor [TNF], interleukin [IL]-1, -6, and -8), arachidonic acid metabolites (prostaglandins, platelet-activating factor, and leukotrienes), proteolytic and lipolytic enzymes; these substances 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 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.[5–10]

In conclusion, we can speculate that various autoimmune and apoptotic sequelae induced by *H. pylori* appear to influence the pathophysiology of AIP, thereby suggesting an underlying link between *H. pylori* infection and AIP. This type of hypothesis should serve as a stimulus for designing a clinical experiment to investigate both the involvement of this bacterium in the pathophysiology of AIP and whether eradication of *H. pylori* infection could indirectly offer benefit to the AIP patients by ameliorating the autoimmune sequelae and the apoptotic loss of duct cells and/or acinar pancreatic cells.

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Less *H. Pylori* Not Necessarily More GERD

TO THE EDITOR: We read with considerable interest Pandolfino *et al.*'s editorial on the impact of *Helicobacter pylori* (*H. pylori*)-associated antral and corpus predominant pattern of gastritis on gastroesophageal reflux disease (GERD) (1). However, this pattern might be confined only to *H. pylori* gastritis acid burden sequelae on GERD, thereby not entailing the overall *H. pylori* repertoire on GERD.

H. pylori gastritis is frequently multifocal throughout the stomach with variable atrophic changes, intestinal metaplasia, and acid burden sequelae, commonly observed by endoscopy and histology, at least in Greece. There is a wide geographic variation in the types of *H. pylori* gastritis, whose clinical consequences and variation cannot be explained by differences in the duration of *H. pylori* infection and virulence (2). Moreover, the factors other than pH responsible for GERD initiation and perpetuation, include deconjugated bile salts and pancreatic enzymes accompanied by a variety of mast cell-derived mediators (release of preformed mediators (histamine, proteoglycans, serine proteases) and *de novo* production of mediators (cytokines, prostaglandins, leukotrienes)) and abnormalities of esophageal peristalsis and/or blunting of the esophagosalivary reflex.

Importantly, the increasing incidence of GERD complications may be explained not just by the diminishing prevalence of *H. pylori* infection as proposed (1), but rather by healing of *H. pylori* associated peptic ulcer disease, which coexists with GERD (3). Thus, eliminating peptic ulcer disease unmasks GERD (3). Our recent data show that *H. pylori* is frequent in GERD and that *H. pylori* eradication leads to better control of GERD symptoms and improves esophagitis (3). *H. pylori* may contribute to GERD pathogenesis by several mechanisms. *H. pylori*, frequently colonizing the gastric cardia as well, induces the release of several mediators (cytokines and nitric oxide), which may adversely affect the lower esophageal sphincter (LES) and induce an inflammatory response, leading to injury of the adjacent esophageal mucosa. Direct damage of the esophageal mucosa can also be caused by strain-dependent bacterial products like cytotoxins, phospholipases, and ammonia derivatives. Additionally, *H. pylori* produces prostaglandins that sensitize afferent nerves and reduce LES pressure. *H. pylori* antrum predominant gastritis is accompanied by an increased gastrin release, thereby augmenting acidity that may exacerbate GERD. Finally, gastric motor function and pyloric sphincter abnormalities in *H. pylori* infected patients (4) may affect the compliance of the gastric wall, favoring GERD, by increasing the gastroesophageal pressure gradient.

We agree with the editorial that *H. pylori* is predominantly linked to noncardia gastric adenocarcinoma, currently a much more pressing public health concern for the developing nations than esophageal adenocarcinoma. However, even in the comparatively rare cases of esophageal adenocarcinoma, our findings indicate a possible pathogenic involvement of *H. pylori* in GERD and inferably in esophageal adenocarcinoma. Therefore, *H. pylori* eradication may reduce the risk of developing not only gastric adenocarcinoma but also GERD-associated esophageal adenocarcinoma at least in the Greek and possibly in other populations exhibiting a similar *H. pylori* pathogenetic response. In this regard, cell cycle response is similar both in short segment Barrett's esophagus (GERD) and in intestinal metaplasia at the gastroesophageal junction (*H. pylori*), reflecting a similar malignant potential (5).

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PostScript

LETTERS

Pitfalls in diagnosing acute pancreatitis

We thank Jolobe for his letter (*Gut* 2005;**54**:1207–8) which mentions the difficulty of establishing the diagnosis of acute pancreatitis in patients with diabetic ketoacidosis.

We did not address diagnostic tests and medical intensive care treatment of acute pancreatitis in our article,[1] but reviewed the interventional and surgical treatment strategies in acute pancreatitis. There is no doubt that treatment of acute pancreatitis, including organ failure in its early phase, is solely supportive.[2] Due to improvements in intensive care medicine, mortality of severe disease has decreased dramatically over the past decades. However, to treat patient adequately, the correct diagnosis has to be made. Therefore, the first step in the diagnostic process of acute pancreatitis is to think of this disease. According to the latest classification from Atlanta,[3] acute pancreatitis is an acute inflammatory process of the pancreas with variable involvement of other regional tissues or remote organ systems. From a clinical point of view, acute upper abdominal pain and elevated pancreatic enzyme levels are needed to diagnose acute pancreatitis. As pointed out by Jolobe in his letter, acute pancreatitis is still underdiagnosed under certain clinical conditions, including diabetic ketoacidosis, but also in other clinical situations, such as shock of unknown origin, patients under intensive care treatment, as well as rare causes of the disease. As acute pancreatitis can be associated with diabetic ketoacidosis and the association between these two is a two way cause-and-effect relationship, early imaging of the pancreas is recommended in these patients to establish the correct diagnosis.

Our article focused on the surgical and interventional treatment of severe acute pancreatitis.[1]

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Severe recurrent Crohn's disease of the ileocolonic anastomosis disappearing completely with antibacterial therapy

Intestinal bacteria play an important role in the pathogenesis of Crohn's disease, which occurs at sites with the highest concentrations of bacteria. Adherent invasive *Escherichia coli* associate with the ileal mucosa in Crohn's disease,[1] and *E coli*, *Bacteroides*, and fusiform bacteria are associated with early disease recurrence after surgical resection.[2] The incidence of clinical recurrence is approximately 50% at three years, with the risk higher in smokers than non-smokers. Endoscopic recurrence occurs in 73–90% of patients one year after surgery,[3] and clinical disease correlates reasonably well with the endoscopic score.[4] Current postoperative prophylaxis therapies are unsatisfactory. Mesalazine 3 g daily, 6-mercaptopurine 50 mg daily, and azathioprine 2 mg/kg daily are only slightly more effective than placebo.[5] Antimicrobial agents have demonstrated efficacy in postoperative prophylaxis reducing the recurrence at the ileocolonic anastomosis. Metronidazole reduced severe endoscopic recurrence at three months and reduced clinical relapse at one year, but was poorly tolerated with significant nausea and vomiting.[6] Ornidazole 1 g daily for one year postoperatively reduced severe endoscopic and clinical recurrence at one year but not at two or three years.[7] We report a case of severe recurrent ileocolonic disease successfully treated with combination ciprofloxacin and metronidazole.

A 23 year old man with a three month history of abdominal pain and diarrhoea was diagnosed with Crohn's disease in October 1996 after a small bowel series revealed narrowing and ulceration of the distal 10 cm of the terminal ileum. His symptoms were steroid responsive for two years. Colonoscopy in June 1998 for recurrent abdominal pain showed severe ulceration and stenosis of the ileocaecal valve and with his symptoms unresponsive to corticosteroids he underwent surgical resection of a segment of severe fistulising terminal ileal Crohn's disease. The remaining small bowel appeared normal. Histopathology confirmed severe active cicatrising Crohn's disease, with stenosis and gross wall thickening with a cobblestone appearance. He made an uncomplicated recovery and remained well without medication. Follow up colonoscopy in April 1999 demonstrated minor recurrent Crohn's disease affecting one third of the ileocolonic anastomosis. He commenced metronidazole 400 mg daily, and on colonoscopy in March 2000 had severe inflammation and narrowing of the entire circumference of the ileocolonic anastomosis preventing ileal intubation. Ciprofloxacin 750 mg/day was added to the metronidazole. Colonoscopy in August 2001 showed superficial ulceration of less than one third of the anastomosis, minimal narrowing, and a normal neoterminal ileum and colon. He continued metronidazole 400 mg daily and ciprofloxacin 750 mg alternate daily until January 2005 when colonoscopy demonstrated a normal anastomosis, neoterminal ileum, and colon. The patient remained on ciprofloxacin 750 mg twice weekly and metronidazole was ceased. Colonoscopy 12 months later showed very minor ulceration affecting 5 mm of the anastomosis, no significant narrowing, and a normal neoterminal ileum.

Ciprofloxacin, a fluoroquinolone, is effective against intestinal aerobic Gram negative bacteria such as *E coli*, *Shigella*, *Salmonella*, and clostridial species. It is well tolerated even with prolonged use[8] but can cause hepatotoxicity and tendon fragility. In Crohn's disease trials, ciprofloxacin was superior to placebo in decreasing the Crohn's disease activity index in one study[9] but ciprofloxacin and metronidazole in combination with budesonide showed no benefit over placebo and budesonide. However, subgroup analysis by disease site revealed a clear improvement in the antibiotic treated group with Crohn's colitis.[10]

To our knowledge, this is the first report of antibacterial therapy producing complete endoscopic resolution of severe postoperative recurrence at the ileocolonic anastomosis. This patient has remained well, and has not developed any untoward side effects. We believe a controlled trial should be performed to test whether low dose ciprofloxacin alone or in combination can prevent recurrence of Crohn's disease at the ileocolonic anastomosis.

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et al, the cells do not have the morphological aspect of a tumour cell and also in other papers on micrometastases, morphology of the cells is not taken into account. In our data set, we could not detect cells in the bone marrow samples which had the morphological aspect of a tumour cell.

With current techniques, epithelial-like cells can be detected in the bone marrow of many patients not known to have cancer. Probably these cells have no clinical relevance in patients with adenomas. In future studies, more attention should be paid to the morphological criteria of the cells.

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Symptomatic eosinophilic gastritis cured with *Helicobacter pylori* eradication

Eosinophilic gastroenteritis is a disorder of unknown aetiology, characterised by eosinophilic infiltration of the gastric and intestinal mucosa and peripheral eosinophilia. To date, only two cases have reported the coexistence of *Helicobacter pylori* gastritis and eosinophilic gastroenteritis. In none of these cases was a causal association between these two entities documented.[1 2] We present a case of eosinophilic gastritis cured with *H pylori* eradication therapy.

A 44 year old woman presented with a two month history of vomiting, nausea, and crampy abdominal pain. Her past medical history included idiopathic thrombocytopenic purpura, splenectomy at the age of 11 years, hyperlipidaemia, hypertension, diabetes mellitus type II, and depression. Her medication included valsartan, atenolol, nifedipine, gliclazide, metformin hydrochloride, indapamide, and venlafaxine hydrochloride. No food allergy or intolerance was reported.

She lived on a Greek island and did not smoke or drink alcohol. Physical examination revealed only mild diffuse epigastric and abdominal tenderness, and no signs of systemic vasculitis or connective tissue disease were present. Laboratory evaluation revealed a white blood cells count of 14 900 cells/mm^3 (normal 4000–10 000) with an eosinophilic count of 2660 cells/mm^3 (normal 0–400), and a normal platelet count (187 000/mm^3) and haemoglobin level (12.8 g/dl). Serum glucose was 158 mg/dl (normal 70–105), alanine aminotransferase 58 mU/ml (normal 10–28), γ-glutamyl transferase 44 mU/ml (normal 9–50), erythrocyte sedimentation rate 29 mm, and C reactive protein 9.19 mg/l (normal 0.00–6.00). Stool studies for ova and parasites and stool cultures were negative. Thyroid function tests, serum IgE, abdominal ultrasound, and computed tomography scan were normal. No findings of extra-abdominal malignancy were evident. Colonoscopy with terminal ileoscopy was normal, as was histology of the ileum and colon. Gastroscopy showed small erosions at the corpus, the antral mucosa, and the duodenal bulb. Biopsies were taken from the gastric corpus, antrum, and from the second part of the duodenum. Gastric biopsies showed *H pylori* infection and dense eosinophilic infiltration (29 eosinophils per high power field) of the gastric mucosa, but biopsies from the 2nd–3rd parts of the duodenum were normal.

She was discharged with a diagnosis of eosinophilic gastritis and *H pylori* erosive gastritis, and was treated with esomeprazole (40 mg/day), amoxycillin (1 g/day), and clarithromycin (1 g/d) for seven days. No treatment was given for eosinophilia. There were no known reports implicating her medications in the aetiology of her symptoms and so it was decided that she should remain on her regular medical treatment. Two months after completion of the eradication therapy repeat gastroscopy was normal. Gastric biopsies showed no *H pylori* infection and only trivial (five eosinophils per high power field) eosinophilic infiltration. She was symptom free, with a white blood count of 3200 cells/mm^3 and a normal eosinophilic count of 780 cells/mm^3. No significant change in platelet count was noted.

Ours is the third reported case of the coexistence of *H pylori* infection and eosinophilic gastroenteritis. In contrast with the previous cases, blood and tissue eosinophilia resolved completely after successful eradication of *H pylori* infection.

Although no confirmed association between *H pylori* gastritis and eosinophilic gastroenteritis can be documented in the literature, our case shows that *H pylori* may play a pathogenic role in the development of blood eosinophilia and eosinophilic gastroenteritis and that *H pylori* eradication may be of value in treating certain cases of this rare syndrome.

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Uneventful pregnancy and neonatal outcome with tacrolimus in refractory ulcerative colitis

Tacrolimus is currently approved only in patients receiving allogeneic liver or kidney transplants.[1] We and others have demonstrated its successful use in refractory colitis.[2 3] Here we report the first patient who was successfully maintained in remission during pregnancy and delivered a healthy baby.

Our patient was diagnosed with ulcerative colitis at the age of 28 years. Her first baby was delivered by Caesarean section prematurely at 29 weeks' gestation. Frequent flare ups of her pancolitis required repeated steroid rescue and she was soon started on azathioprine. Unfortunately, she was unable to tolerate purine analogues due to heterozygous thiopurine methyltransferase deficiency causing severe pancytopenia and life threatening sepsis.

Discussing the remaining therapeutic options, the patient refused proctocolectomy and ileoanal pouch anastomosis and chose a trial on oral tacrolimus. She was started on 0.1 mg/kg body weight oral tacrolimus, divided into two daily doses. The dose was adjusted aiming for serum trough levels of 4–6 ng/ml. Her condition improved quickly within the following weeks and complete remission was achieved. The patient was discouraged from conceiving while on tacrolimus. When weaning off tacrolimus resulted in repeated disease flare ups, she underwent granulocyte apheresis (Adacolumn) at age 37 years but was unable to attain prolonged remission without tacrolimus.

At the age of 41 years she became intentionally pregnant with her second child. Sonographic malformation screening during the second trimester did not detect any fetal abnormalities. She spontaneously delivered a healthy baby girl (Apgar score 9/10/10; birth weight 3500 g; height 51 cm) at 40 weeks' gestation. She was continued on tacrolimus throughout the pregnancy and following delivery, aiming for serum trough levels of 4–6 ng/ml, and maintained a stable remission. To date she is 33 years old and still in remission. Because of the unknown immediate and long term side effects of tacrolimus in the newborn, we recommended refraining from breastfeeding. There is no evidence of any functional impairment or developmental delay in her now two year old daughter.

This case is the first report of tacrolimus use during pregnancy for refractory ulcerative colitis. Most experience with tacrolimus in pregnancy exists with transplant patients. In a recent study, 17 female liver transplant recipients who delivered 19 babies were reported. Thirty six mothers (97%) survived the pregnancy. One patient who showed an intra-aortic arterial graft during labour died. The mean gestational period was 36.4 ± 3.2 weeks, excluding two premature