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Alimentary Tract

Bleeding duodenal ulcer: comparison between *Helicobacter pylori* positive and *Helicobacter pylori* negative bleeders

A.B. Adamopoulos*, S.P. Efstathiou, D.I. Tsioulos, D.G. Tzamouranis, A.G. Tsiakou, Dina Tiniakos, T.D. Mountokalakis

Third Department of Internal Medicine, University of Athens, Medical School, Sotiria General Hospital, Building Z, Mesogion 152, 11527 Athens, Greece

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Abstract

Background and aims. To provide a direct comparison of *Helicobacter pylori*-positive subjects bleeding from duodenal ulcer with *H. pylori*-negative ones, in terms of severity of bleeding and outcome.

Patients and methods. A case-control study was prospectively conducted in 105 *H. pylori*-negative duodenal ulcer bleeders and same number of sex- and age-matched *H. pylori*-positive ones.

Results. NSAID consumption was more common among *H. pylori*-negative subjects (81%) compared to their *H. pylori*-positive counterparts (58.1%, $P < 0.001$). *H. pylori*-negative bleeders were found to need more often haemostasis (55.2% versus 31.4%, $P < 0.001$) or surgical intervention (15.2% versus 4.8%, $P = 0.011$) and to have a greater proportion of rebleeding (32.4% versus 13.3%, $P = 0.001$), a more prolonged hospitalisation (11.6 ± 4.1 versus 6.2 ± 1.5 days, $P < 0.001$) and a higher rate of in-hospital mortality (15.2% versus 3.8%, $P = 0.005$). In the overall population ($N = 210$), *H. pylori* negativity, among other known risk factors, emerged as independent predictor (odds ratio 3.2; 95% CI: 1.5, 11.2; $P = 0.004$) of an unfavourable outcome (surgery or death).

Conclusions. Duodenal ulcer bleeding in *H. pylori*-negative subjects appears to be more severe, to have a higher rate of rebleeding, and to lead more often to surgery or fatality compared to the vast majority of *H. pylori*-positive duodenal ulcer bleeders.

Keywords: *H. pylori* infection; Peptic ulcer; Upper gastrointestinal bleeding

1. Introduction

Acute bleeding from peptic ulcer remains a major cause of morbidity and mortality despite effective medical treatment of peptic ulcer disease [1]. It has been estimated that approximately 3% of patients with duodenal ulcer (DU) who are not receiving anti-secretary therapy are likely to develop haemorrhage during each year of follow-up, with a cumulative risk for bleeding within 5 years of up to 14% [2].

Helicobacter pylori (HP) infection is widely accepted as the most important factor in the pathogenesis of DU [3]. Nevertheless, in parallel with more effective eradication of the organism, the prevalence of the infection is changing and *H. pylori*-negative [HP (−)] peptic ulcer disease appears to be increasing [4,5]. Accumulating evidence suggests that *H. pylori* infection is less frequent in patients with a bleeding DU [6] compared to those with an uncomplicated DU [7]. Numerous case-control studies have evaluated the role of *H. pylori* infection in DU bleeding by assessing the characteristics of DU bleeders as opposed to those of hospital or community controls with uncomplicated DU [8,9]. However, data on potential differences between *H. pylori*-associated DU bleeding and haemorrhage from a DU taking place in the absence of *H. pylori* infection are lacking.

The aim of this case-control study was to provide a direct comparison of *H. pylori*-positive [HP (+)] DU bleeders with *H. pylori*-negative ones, in terms of severity of bleeding and outcome.

2. Materials and methods

2.1. Study design and population

This was a case-control study conducted in Athens, at the Third University Department of Internal Medicine of the

* Corresponding author. Tel. +30 210-7719975; fax: +30-210-7719981.
E-mail address: sefstath@otellas.gr (A.B. Adamopoulos).

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'Sotiria' General Hospital. From April 1995 to April 2003, all patients who were admitted at the Emergency Department with clinical manifestations of upper gastrointestinal haemorrhage were considered for entry in the study. Data from all the *H. pylori*-negative subjects of the above series with bleeding DU confirmed endoscopically were prospectively recorded. Only subjects bleeding solely from DU were studied, while patients with DU and additional lesions (gastric ulcer, erosions etc.) were discarded. Each *H. pylori*-negative DU bleeder was matched with a control one. Eligible controls were *H. pylori*-positive DU bleeders, matched for age (5-year bands) and sex, who were consecutively enrolled in the study population, according to the date of admission, in order to avoid selection bias. All study participants were outpatients. The primary endpoint was the investigation of potential differences in outcome, defined as surgery or death, between *H. pylori*-negative and *H. pylori*-positive DU bleeders, whereas comparisons of the two aforementioned groups in terms of clinical characteristics, endoscopic findings, severity of haemorrhage and course during hospitalisation were considered as secondary endpoints.

Exclusion criteria were the following: (i) congestive heart failure grade IV, respiratory failure requiring mechanical ventilation, chronic renal failure on haemodialysis, major neurological deficit, severe haematological derangements or electrolytic aberrations (cirrhotic patients were not excluded unless they had considerable coagulopathy or hepatic encephalopathy); (ii) previous intake of antibiotics or proton pump inhibitors (PPIs) during the last month before the bleeding episode; (iii) history of gastric surgery or of *H. pylori* eradication therapy; (iv) strong suspicion of perforation; (v) death or surgery before endoscopy.

2.2 Data collection

Data collection was carried out by use of a structured form including a standardised questionnaire. Haemodynamic instability was defined as: SBP < 100 mmHg and/or HR > 100 min^{-1}, and/or orthostatic changes in SBP (decrease of >10%) or in HR (increase of >10%) between supine and mostly sitting (than upright) position. Patients were considered as NSAID users if they reported ingestion of at least one dose of NSAID during the last 4 weeks before admission. NSAID consumers were subgrouped as follows: occasional users (sporadic, as needed during the previous week), acute users (continuous therapy for less than 1 month) and chronic users (continuous therapy for more than 1 month).

2.3 Endoscopic interventions

All study participants underwent upper gastrointestinal endoscopy by the same endoscopist within 24 h from admission. Urgent endoscopy within 12 h from admission was decided on the basis of individual patients' overall assessment by using data from initial evaluation at the Emergency Department [10]. The equipment used was the Fuginon FG-100 (Fuji Photo Optical Co. Ltd., Saitama city, Japan). An ulcer was defined as a circumscribed mucosal break with an apparent depth and a diameter of at least 5 mm. Patients with DU as the only cause of bleeding were included in the study. Biopsies were obtained at the time of initial endoscopy when the patient's condition allowed some minutes of delay; otherwise the procedure was postponed for 2 days later.

Ulcer lesions were divided into six categories according to a modified Wara's classification [11]: (a) bleeding visible vessel, either spurting or oozing, (b) non-bleeding visible vessel, (c) adherent clot, (d) oozing (without visible vessel), (e) red/black spot and (f) clean base. Active bleeding was defined as the presence of a bleeding visible vessel, either spurting (Forrest IA) or oozing (Forrest IB), or of an oozing (Forrest IB) from the ulcer base [12] without a visible vessel. Haemostatic endotherapy with local injection of epinephrine solution 1:10,000 was performed in all cases of active bleeding. Haemostatic stabilisation with adrenaline was performed in ulcers with non-bleeding visible vessels (or protuberance) as well. Follow-up endoscopy was performed after 48 h in patients who had stigmata of recent bleeding or received endoscopic therapy, as well as in those who had signs of rebleeding.

In cases where biopsies for culture, histology and rapid urease test were taken at the initial endoscopy, PPIs were administered after the ^{13}C-urea breath test was performed, that is, as soon as oral feeding was allowed after the bleeding episode. In regard to subjects who had stigmata of recent bleeding and those who received endoscopic therapy, no biopsies were initially taken and no PPIs were administered after the first endoscopy. As soon as the second look endoscopy was performed, biopsies were obtained, oral feeding was allowed and the ^{13}C-urea breath test was carried out, they were also given PPIs. In all cases, no PPIs or H2 blockers were administered until all tests for the assessment of *H. pylori* status had been performed.

2.4. Diagnosis of H. pylori infection

H. pylori infection was tested in all cases by histology, rapid urease test, ^{13}C-urea breath test and culture. All the above four tests were performed for each subject, since no patient was directly led to surgery or died before endoscopy (exclusion criteria). Subjects were considered infected if any of the four diagnostic modalities gave a positive result and non-infected if all four tests were negative [4]. Diagnostic tests for identifying *H. pylori* were performed as follows:

(1) Histological study was performed on two antral and two body samples, which were fixed in 10% formalin, embedded in paraffin and stained with haematoxylin-eosin and modified Giemsa stain. All specimens were examined by the same pathologist who was blinded to the endoscopic findings and the results of the other diagnostic methods. A sample was considered positive when the pathologist

observed the presence of bacillus of helicoidal morphology adhering to the surface epithelium of the gastric mucosa.

(II) A sample of mucosa from gastric antrum obtained during endoscopy was immediately introduced into a commercially available diagnostic media for rapid urease test (Kimberly–Clark CLO test, Ballard Medical Products; Draper, Utah, USA) and incubation was maintained for 24 h. According to the manufacturer's instructions, a change of colour from the initial yellow to pink or orange was considered positive.

(III) A ^{13}C-urea breath test (INFAI ^{13}C-urea breath test; Bochum, Germany) was carried out in all patients as soon as oral feeding was allowed after the bleeding episode. The ^{13}C-urea breath test was performed according to a standardised protocol, the sensitivity and specificity of which are approximating 100% [13]. An increase in $^{13}CO_2$ in breath was detected by isotope-ratio-mass-spectrometry (IRMS) and expressed as absolute difference between the 30 min and the 0 min value. The test indicated the presence of *H. pylori* if the increase of $^{13}CO_2$ exceeded the difference value of 4‰.

(IV) Two antral biopsies were transported to the microbiology department within an intended period of not more than 12 h in modified Stuart transport agar. The agar plates were incubated at 37 °C in microaerobic conditions (Campy Pak Plus, BBL; Becton Dickinson, Cockesville) and read after 7 days.

2.5. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS Inc., Chicago, Illinois, release 10.0). Comparisons between the two groups were made by Student's paired *t*-test for continuous variables and by chi-square test or Fischer's exact test, as appropriate, for categorical data. Using data from the total study population, univariate correlations were performed between patients' characteristics and the presence of an adverse outcome, defined as surgery or death. Variables with a significant univariate association with an unfavourable outcome were considered in a multiple logistic regression analysis using a forward stepwise selection algorithm with entry and removal criteria of $P = 0.05$ and 0.10, respectively. In the multivariate procedure, the presence of surgery or death was used as the end point. Ninety-five percent confidence intervals (95% CI) were calculated for each comparison. All tests of significance were two-tailed and a P value less than 0.05 was considered to be significant.

3. Results

During the study period, 656 subjects with DU bleeding were assessed, 105 of whom (16%) were considered *H. pylori*-negative according to the aforementioned criteria. These subjects were compared to 105 sex- and age-matched consecutive *H. pylori*-positive controls from the same series, the case-control study population consisting of 210 DU bleeders. Patients' characteristics with regard to *H. pylori* status are shown in Table 1. No difference was found between the two groups in terms of history of peptic ulcer or ulcer bleeding, alcohol abuse, smoking habits, comorbidity, comedication (apart from NSAIDs), clinical manifestations and laboratory features on admission (Table 1).

On the basis of the presence of haemodynamic instability and fresh blood in the nasogastric tube, of the number of blood units transfused and of the initial haemoglobin levels, the bleeding episode was more severe in *H. pylori*-negative bleeders (Table 1). The latter subjects were also found to need more often haemostasis or hamostatic stabilisation than their *H. pylori*-positive counterparts (55.2% versus 31.4%, $P < 0.001$) and to have a greater proportion of rebleeding (32.4% versus 13.3%, $P = 0.001$) (Table 1). Further, *H. pylori*-negative patients had a more prolonged hospitalisation (11.6 ± 4.1 days) compared to *H. pylori*-positive ones (6.0 ± 1.6 days, $P < 0.001$), were led to surgery more often (15.2% versus 4.8%, $P = 0.011$) and had a higher rate of in-hospital mortality (15.2% versus 3.8%, $P = 0.005$, Table 1). In both groups, all surgeries were performed and all deaths occurred after rebleeding. The same proportion of death during surgery was observed in the two groups (2 out of 105, 1.9%), whereas overall mortality rate was 9.5%.

The diagnostic value of the four tests used for the diagnosis of *H. pylori* infection is presented in Table 2. The pattern of NSAID consumption among the two groups is shown in Table 3. A greater proportion of *H. pylori*-negative subjects reported NSAID intake (81%) compared to *H. pylori*-positive patients (58.1%, $P < 0.001$), whereas the rate of aspirin use did not differ significantly between the two groups (47.6% versus 35.2%, respectively, Table 3). However, the difference found between the two groups in the proportion of aspirin intake (>10%) seems clinically relevant, suggesting the occurrence of a *beta* error, probably due to the low statistical power for the latter comparison. The remaining aspects of NSAID consumption, including duration of usage, proportion of multiple or OTC consumption, indication for use, route of administration, dosage and type of intake (i. e., acute, occasional or chronic) did not differ between *H. pylori*-negative and *H. pylori*-positive bleeders (Table 3). Non-NSAID related *H. pylori*-negative DU bleeders accounted for 19% of *H. pylori*-negative subjects (20 out of 105), that is, 3.1% of all DU bleeders ($N = 656$) evaluated during the study period.

Endoscopic findings are presented in Table 4. *H. pylori*-positive subjects were found to have more frequently a DU with no stigmata of recent haemorrhage (34.3%) as opposed to *H. pylori*-negative bleeders (21%, $P < 0.05$). Nevertheless, *H. pylori*-negative bleeders had an overall higher rate of active bleeding in endoscopy (40% versus 20%, $P = 0.002$) (Table 4).

Table 1
Demographic, clinical and laboratory characteristics according to *H. pylori* status (OR, odds ratio; 95% CI, 95% confidence intervals). For continuous variables, data indicate mean ± standard deviation or mean difference ± standard deviation

Patients' characteristics	*H. pylori*-positive bleeders (%)	*H. pylori*-negative bleeders (%)	*P* value	OR	95% CI
Demographic data					
Male sex	87 (66.9)	87 (66.9)			
Age (years)	65.0 ± 14.9	64.3 ± 16.7	0.901	0.7 ± 1.3	2.0, 2.4
History					
Alcohol abuse	28 (26.7)	22 (21)	0.331	0.7	0.4, 1.4
Current smoking	49 (46.7)	52 (49.5)	0.678	1.1	0.7, 1.9
History of peptic ulcer	16 (15.2)	11 (10.5)	0.302	0.7	0.3, 1.5
History of ulcer bleeding	6 (5.7)	4 (3.8)	0.516	0.7	0.2, 2.4
Comorbidity					
Coronary heart disease	25 (23.8)	20 (19)	0.401	0.8	0.4, 1.5
Congestive heart failure	5 (4.8)	3 (2.9)	0.470	0.6	0.2, 2.5
Cerebrovascular disease	3 (2.9)	2 (1.9)	0.650	0.7	0.1, 4.1
Chronic obstructive pulmonary disease	6 (5.7)	9 (8.6)	0.421	1.5	0.5, 4.5
Chronic renal insufficiency	9 (8.6)	8 (7.6)	0.800	0.9	0.3, 2.4
Cirrhosis	12 (11.4)	11 (10.5)	0.825	0.9	0.4, 2.2
Malignancy	6 (5.7)	7 (6.7)	0.774	1.2	0.4, 3.6
Bedridden status	6 (5.7)	7 (6.7)	0.774	1.2	0.4, 3.6
Comedication					
Beta blockers	17 (16.2)	12 (11.4)	0.317	0.7	0.3, 1.5
Calcium channel blockers	13 (12.4)	10 (9.5)	0.507	0.8	0.3, 1.8
Corticosteroids	2 (1.9)	2 (1.9)			
Anti-coagulants	2 (1.9)	1 (1)	0.560	0.5	0.1, 5.5
Clinical manifestations at presentation					
Hematemesis	10 (9.5)	6 (5.7)	0.298	0.6	0.2, 1.7
Melaena	62 (59)	70 (66.7)	0.253	1.4	0.8, 2.4
Coffee grounds vomiting	8 (7.6)	5 (4.8)	0.390	0.6	0.2, 1.9
Combination of above	25 (23.8)	24 (22.9)	0.870	0.9	0.5, 1.8
Severity of UGIB					
Initial haemoglobin (g/dl)	10.5 ± 2.6	8.2 ± 2.1	<0.001	2.3 ± 0.7	1.4, 3.0
Hemodynamic instability on admission	20 (19.1)	46 (43.8)	<0.001	3.3	1.8, 6.2
Fresh blood in the nasogastric tube	13 (12.4)	40 (38.1)	<0.001	4.4	2.2, 8.8
Blood units transfused	2.2 ± 1.6	3.7 ± 2.2	0.003	1.5 ± 0.9	1.1, 2.6
Urgent endoscopy	59 (56.2)	80 (76.2)	0.002	2.5	1.4, 4.5
Active bleeding	21 (20)	42 (40)	0.002	2.7	1.4, 4.9
Need for haemostasis or stabilisation	33 (31.4)	58 (55.2)	<0.001	2.7	1.5, 4.7
Laboratory features at presentation					
White blood cells (μl)	10885 ± 4900	10507 ± 3530	0.703	378 ± 950	−1550, 2460
Platelets (μl)	249900 ± 114773	246400 ± 80788	0.897	3500 ± 25860	−55130, 47720
Prothrombin time (s)	14.5 ± 2.6	14.1 ± 1.6	0.421	0.4 ± 0.4	0.6, 1.4
Partial thromboplastin time (s)	30.4 ± 6.4	29.7 ± 6.7	0.801	0.7 ± 1.5	3.0, 2.7
Urea (mg/dl)	82.1 ± 50	74.5 ± 39	0.402	7.6 ± 8.5	−29.2, 14.2
Course					
Days of hospitalisation	6.0 ± 1.6	11.6 ± 4.1	<0.001	5.6 ± 1.2	2.9, 7.9
Rebleeding	14 (13.3)	34 (32.4)	0.001	3.1	1.6, 6.2
Surgery	5 (4.8)	16 (15.2)	0.011	3.6	1.3, 10.2
Death	4 (3.5)	16 (15.2)	0.005	4.5	1.5, 14.1

Table 2
The diagnostic value of the four tests used for the diagnosis of *H. pylori* infection (PPV, positive predictive value; NPV, negative predictive value)

Diagnostic test	Sensitivity	Specificity	PPV	NPV	Accuracy
Rapid urease test	51.4 (34.5–70.1)	100 (64.1–100)	100 (85–100)	27.8 (14.9–40.2)	58.3
Breath test	93.2 (80.1–97.2)	74.4 (40.1–90.5)	94 (83.3–98.1)	60.6 (34.4–81.5)	89.2
Culture	55.2 (37.2–76.9)	100 (64.6–100)	100 (87–100)	29.6 (18.3–47.8)	59.7
Histology	82.2 (64.8–89.4)	100 (65.2–100)	100 (91–100)	51.5 (26.6–70.7)	84.2

Table 3
NSAID use pattern among *H. pylori*-positive and *H. pylori*-negative bleeders from duodenal ulcer (OR, odds ratio; 95% CI, 95% confidence intervals) For continuous variables, data indicate mean ± standard deviation or mean difference ± standard deviation

NSAID consumption	*H. pylori*-positive bleeders (%)	*H. pylori*-negative bleeders (%)	*P* value	OR	95% CI
Overall NSAID intake	61 (58.1)	85 (81)	<0.001	3.1	1.7, 5.7
Aspirin (including enteric coated)	37 (35.2)	50 (47.6)	0.069	1.7	0.9, 2.9
Non-aspirin NSAIDs	24 (22.9)	35 (33.3)	0.091	1.7	0.9, 3.1
Duration of use before haemorrhage (days)	77.1 ± 48.2	70.1 ± 69.3	0.965	7.0 ± 8.5	16.5, 28.7
Multiple NSAID use	15 (14.3)	20 (19.1)	0.354	1.4	0.7, 2.9
Over the counter use	29 (27.6)	37 (35.2)	0.234	1.4	0.8, 2.6
Indication					
Rheumatic disease	7 (6.7)	12 (11.4)	0.229	1.8	0.7, 4.8
Non-specific arthralgias	10 (9.5)	16 (15.2)	0.209	1.7	0.7, 4.0
Headache	10 (9.5)	15 (14.3)	0.286	1.6	0.7, 3.7
Lower back pain	10 (9.5)	13 (12.4)	0.507	1.3	0.6, 3.2
Cardiovascular disease	22 (21)	27 (25.7)	0.414	1.3	0.7, 2.5
Miscellaneous	2 (1.9)	2 (1.9)		-	-
Route of administration					
Oral	40 (38.1)	53 (50.5)	0.070	1.7	0.9, 2.9
Parenteral	12 (11.4)	15 (14.3)	0.536	1.3	0.6, 2.9
Rectal	9 (8.6)	17 (16.2)	0.093	2.1	0.9, 4.9
Type of consumption:					
Acute	13 (12.4)	16 (15.2)	0.548	1.3	0.6, 2.8
Occasional	28 (26.7)	40 (38.1)	0.076	1.7	0.9, 3.0
Chronic	20 (19.1)	29 (27.6)	0.142	1.6	0.9, 3.1
Dosage					
Low	20 (19.1)	29 (27.6)	0.142	1.6	0.9, 3.1
Medium	21 (20)	32 (30.5)	0.080	1.8	0.9, 3.3
High	20 (19.1)	24 (22.9)	0.498	1.3	0.7, 2.5

Using data from the total case-control study population ($N = 210$), univariate correlations were performed between patients' characteristics and the presence of an adverse outcome (defined as surgery or death in hospital), accounting overall for 41 subjects (19.5%). Age ($P < 0.001$), comorbidity ($P < 0.001$), haematemesis as a presenting symptom ($P < 0.05$), fresh blood in the nasogastric tube ($P < 0.01$), haemodynamic instability on admission ($P < 0.001$), initial haemoglobin level ($P < 0.001$), number of blood units transfused ($P < 0.05$), active bleeding in endoscopy ($P < 0.01$), rebleeding ($P < 0.001$) and *H. pylori* negativity ($P < 0.001$) were found to be significantly associated with an unfavourable outcome.

When the above 10 variables were entered in the multivariate model, age >60 years, comorbidity, haemodynamic instability on admission, initial haemoglobin < 8 g/dl, rebleeding and *H. pylori* negativity emerged as independent predictors of an adverse outcome (Table 5). The same variables as above were isolated as significant univariate and multivariate correlates even when only in-hospital death was considered as the end point in the analysis. Thus, *H. pylori* negativity (odds ratio (OR): 2.9; 95% CI: 1.3, 16.1; $P = 0.009$) remained independently associated with mortality, among age >60 years (OR: 8.3; 95% CI: 3.1, 43.5; $P < 0.001$), comorbidity (OR: 13.6; 95% CI: 4.4, 56.8; $P < 0.001$), haemodynamic instability on admission (OR: 0 4.2; 95% CI: 2.0, 20.6; $P = 0.003$), initial haemoglobin < 8 g/dl (OR: 6.3; 95% CI: 2.5, 28.9; $P < 0.001$) and rebleeding (OR: 15.2; 95% CI: 4.8, 51.5; $P < 0.001$).

Table 4
Endoscopic findings in *H. pylori*-positive and *H. pylori*-negative bleeders from duodenal ulcer (OR, odds ratio, 95% CI, 95% confidence intervals)

Endoscopic findings	*H. pylori*-positive bleeders (%)	*H. pylori*-negative bleeders (%)	*P* value	OR	95% CI
Ulcer with clear base	36 (34.3)	22 (21)	0.031	2.0	1.1, 3.7
Ulcer with red/black spot	32 (30.5)	21 (20)	0.081	1.8	0.9, 3.3
Ulcer with adherent clot	4 (3.8)	4 (3.8)			
Non-bleeding visible vessel	12 (11.4)	16 (15.2)	0.416	1.4	0.6, 3.1
Bleeding visible vessel, spurting (Forrest IA)	4 (3.8)	9 (8.6)	0.152	2.4	0.7, 7.9
Bleeding visible vessel, oozing (Forrest IB)	6 (5.7)	12 (11.4)	0.139	2.1	0.8, 5.9
Oozing from an ulcer base without a visible vessel (Forrest IB)	11 (10.5)	21 (20)	0.060	2.1	0.9, 4.7
Active bleeding (overall)	21 (20)	42 (40)	0.002	2.7	1.4, 4.9

Table 5
Independent predictors of adverse outcome in the overall study population (OR, odds ratio; 95% CI, 95% confidence intervals)

Variables	Overall ($N = 200$) (%)	Adverse outcome ($N = 43$) (%)	P value	OR	95% CI
Age (>60 years)	87 (41.4)	29 (70.7)	<0.001	7.5	3.2, 20.4
Comorbidity	116 (55.2)	36 (87.8)	<0.001	12.4	4.2, 37.3
Haemodynamic instability at admission	66 (31.4)	27 (65.9)	0.002	5.7	2.5, 14.1
Initial haemoglobin < 8 g/dl	72 (34.3)	30 (73.2)	<0.001	8.8	3.4, 28.9
Rebleeding	48 (22.9)	41 (100)	<0.001	17.1	5.8, 55.3
H. pylori negativity	105 (50)	32 (78.1)	0.004	3.2	1.5, 11.2

4. Discussion

Although the role of *H. pylori* infection as a major aetiologic factor for uncomplicated DU disease as well as for DU bleeding is well recognised [3], the prevalence of *H. pylori*-negative DU disease appears to be increasing [4,5]. A randomised trial assessing differences between *H. pylori*-negative and *H. pylori*-positive subjects with uncomplicated DU [4] has shown a poorer clinical outcome, defined as unhealed ulcer and/or symptomatic ulcer recurrence, over a 2-year follow-up period in *H. pylori*-negative patients, especially if treated empirically with eradication therapy. However, data comparing severity of bleeding and/or fatality between *H. pylori*-positive and *H. pylori*-negative DU bleeders are provided for the first time by the present study.

The main findings of this study is that DU bleeding in non-infected subjects seems to be more severe, to be more often complicated with rebleeding and to result more frequently to surgery or death as opposed to *H. pylori*-positive patients. *H. pylori*-negative bleeders were found to have at least a three-fold greater probability for rebleeding, surgery or death compared to their *H. pylori*-positive counterparts, the 95% confidence intervals ruling out a difference smaller than 30% in the probability of an unfavourable course between the two groups. Validation of the above results in other populations is warranted before *H. pylori* negativity is established as an additional factor useful for risk stratification of patients presenting with haemorrhage from a DU. However, it should be stressed that the potential prognostic role of *H. pylori* infection is hampered by the caveat of possible delay (up to 48 h) in a definite diagnosis, especially in subjects with active bleeding or a negative rapid urease test.

The finding of *H. pylori*-negative DU bleeders having a higher rate of rebleeding, which was used as a surrogate marker of poor outcome in the present study, appears to be in divergence with the results of a recent report [14] that has shown no difference in the proportion of ulcer rebleeding between *H. pylori*-negative and *H. pylori*-positive subjects. However, the population of the latter study consisted of both duodenal and gastric ulcer bleeders, the prevalence of *H. pylori*-negative peptic ulcer being as high as 37%. Moreover, the diagnosis of *H. pylori* infection was made upon histology and rapid urease test (rather than histology, urea breath test, rapid urease test and culture) and no case-control design was used.

The present study has evaluated a large population of a relatively small subgroup of patients presenting with haemorrhage from a DU. The prevalence of *H. pylori* infection in the overall population studied was 84% (551 out of 656 DU bleeders), being in accordance with previous reports that have suggested that infection with this organism is less common among patients with bleeding DU (73–88%) [6] compared to those with non-bleeding DU (almost 100%) [15]. This difference has mainly been attributed to misclassification of subjects due to inaccurate diagnosis of *H. pylori* infection [16], the latter not being always detected in ulcer bleeders with the diagnostic methods commonly used in non-complicated ulcer patients [15]. Moreover, recent antibiotics and/or PPIs administration has been shown to induce false-negative results [15]. Taking into account the aforementioned limitations, in order to achieve the optimal diagnostic accuracy in the present study, we decided to use four diagnostic tests to define *H. pylori*-positive and *H. pylori*-negative DU bleeders, to exclude subjects with recent PPIs or antibiotics use and not to administer PPIs to study participants until all tests were performed. The pattern of the diagnostic value of the four tests used in our study provides additional support to current knowledge on the diagnosis of *H. pylori* infection in ulcer bleeders, according to which rapid urease test and culture may give a high rate of false-negative results, whereas histology, rapid urease test and culture have a specificity of 100% [6,17].

A substantial difference in the proportion of NSAID use was found between *H. pylori*-positive and *H. pylori*-negative bleeders, the vast majority of the latter (81%) reporting NSAID intake within 4 weeks prior to the bleeding episode. A recent meta-analysis of case-control studies has suggested that these two risk factors have a synergistic effect on ulcer bleeding [18], whereas a large controlled study has recently shown triple eradication therapy or anti-secretory treatment with omeprazole to reduce the occurrence of NSAID associated peptic ulcer and dyspeptic symptoms requiring therapy [19]. In regard to the impact of NSAID use on *H. pylori*-negative subjects' prognosis in the present study, it appears unlikely that the high rate of NSAID consumption was the determining factor, since only half of *H. pylori*-negative patients who needed surgical intervention (8 out of 16) or died in hospital (8 out of 16 as well) had used NSAIDs. Moreover, multivariate analysis yielded no association between NSAID intake and outcome in the

overall study population, this finding being in agreement with previous reports, which have suggested that NSAID consumption has no effect on prognosis of patients with haemorrhage from an ulcer [20,21]. Further, a recent study directly comparing NSAID and non-NSAID bleeders [22] has been shown that severity of bleeding and outcome do not differ between the two groups.

The pathogenesis of uncomplicated as well as of bleeding DU has not been completely elucidated. Gastric acid hypersecretion, pepsinogen activity, abnormal gastric emptying and *H. pylori*-related mechanisms have been implicated. On the basis of our findings, it may be speculated that a gastric metaplastic island in the duodenum infected by a *H. pylori* strain could stimulate ulcerogenesis [23,24], whereas mucosal defence mechanisms are mobilised leading to the development of a relatively stable ulcerative lesion after a certain time intake. On the other hand, in the absence of a metaplastic *H. pylori* infected island, the derangement of an "unprepared for defence" duodenal mucosa is acute, deeper, more aggressive and more likely to erode larger vessels. In addition, *H. pylori*-negative bleeders may include a subgroup of true acid hyper-secretors with a greater acid load in the duodenum as compared to their *H. pylori*-positive counterparts. Nevertheless, in the cases of *H. pylori*-negative bleeding, the role of NSAIDs may be restricted to that of the triggering but not the determining factor of the severity of the haemorrhage.

The incidence of *H. pylori*-negative non-NSAID related bleeding in the present study was 3.1%, corroborating previous reports [4,5,15,16,18,25], according to which both peptic ulcer disease and ulcer bleeding are rare in *H. pylori*-negative non-NSAID consumers. Although it was impractical to measure serum salicylate and plasma thromboxane levels due to the size of the study, NSAID use in every case has been prospectively scrutinised by a specific questionnaire. However, given that surreptitious NSAID intake cannot absolutely be excluded, the true prevalence of NSAID-related bleeding may still have been underestimated. Interestingly, all 20 *H. pylori*-negative non-NSAID bleeders in the present study had comorbidities predisposing to peptic ulcer disease and haemorrhage [26–28] (10 cirrhosis, 6 chronic renal insufficiency, 4 severe chronic obstructive pulmonary disease with recent hospitalisation because of respiratory failure requiring mechanical ventilation). Hence, it could be suggested that virtually no case of 'truly idiopathic' bleeding DU existed in the population studied. The unfavourable outcome of the above subgroup (40% (8 out of 20) surgical intervention, 40% mortality) probably resulted from the severe coexisting diseases of these subjects (eight cirrhosis, four chronic renal insufficiency, four severe chronic obstructive pulmonary disease).

In conclusion, the novel information provided by the present study is that DU bleeding in *H. pylori*-negative subjects appears to be more severe, to have a higher rate of rebleeding, and to lead more often to surgery or fatality compared to the vast majority of *H. pylori*-positive bleeders. This difference seems unlikely to be associated with NSAID use or other ulcerogenic factors. If confirmed in other populations, these findings outline the potentiality of incorporating the parameter of *H. pylori* status into clinico-prognostic models of ulcer bleeding that may allow for a more accurate identification of high-risk bleeders and optimise their management.

Conflict of Interest Statement

None declared.

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Esomeprazole versus Omeprazole for the Eradication of *Helicobacter pylori* Infection

Results of a Randomized Controlled Study

George K. Anagnostopoulos, MD, Stavros Tsiakos, MD, George Margantinis, MD, Panagiotis Kostopoulos, MD, Dimitrios Arvanitidis, MD, PhD

Background: Esomeprazole has higher oral bioavailability and increased antimicrobial activity against *Helicobacter pylori* than omeprazole.

Goals: To compare 7 days esomeprazole with 7 days of omeprazole based triple therapies for the eradication of *H. pylori*, and to assess whether the administration of higher dose of esomeprazole leads to improved eradication rates.

Study: One hundred and fifty-six dyspeptic patients with *H. pylori* received either: (1) 1-week treatment including esomeprazole 40 mg once daily, amoxicillin 1 g, and clarithromycin 500 mg, both twice daily (EAC1 group, n = 52); (2) 1-week treatment of omeprazole 20 mg, amoxicillin 1 g, and clarithromycin 500 mg, all administered twice daily (OAC group, n = 52); or (3) 1-week treatment with esomeprazole 40 mg, amoxicillin 1 g, and clarithromycin 500 mg, all given twice daily (EAC2 group, n = 52).

Results: *H. pylori* was eradicated in 37 of 52 patients in the OAC group (Intension to treat [ITT] 71%), and in 42 patients in the EAC1 group (ITT 81%). High eradication rate was achieved by the EAC2 regimen (ITT, 96%), but more patients reported unwanted effects.

Conclusion: Seven days of esomeprazole based triple therapy is a satisfactory eradication regimen for *H. pylori* infection. Higher doses of esomeprazole have excellent eradication rates, but they may lead to increased side effects.

Key Words: eradication, helicobacter, esomeprazole, omeprazole

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It is widely accepted that *Helicobacter pylori* infection is an important risk factor for gastric malignancy, and is linked to most cases of both duodenal and gastric ulcer.[1] Although the best regimen for the eradication of *H. pylori* is not yet clear, 1-week triple therapies with a proton pump inhibitor (PPI), clarithromycin, and either amoxycillin or metronidazole, are considered preferable.[2] Recent data suggest that 10- to 14-day triple therapies could achieve higher eradication rates than 7-day schedules,[3] but poor patient compliance and development of adverse side effects may make these long regimens less desirable.[4] The minimum value an eradication therapy should achieve is 80%.[2]

Proton pump inhibitors have 2 potential mechanisms of action in the treatment of *H. pylori*. They inhibit gastric acid secretion, and exert an antimicrobial activity in vitro, against *H. pylori*. Esomeprazole, is the first PPI developed as an optical isomer (L-isomer).[5] Its oral bioavailability is higher than omeprazole resulting in greater acid suppression.[6] It has also been shown that esomeprazole has increased antimicrobial activity against *H. pylori*, in vitro, compared with omeprazole.[7]

The aim of this study is to compare 7 days esomeprazole with 10 days of omeprazole based triple therapies for the eradication of *H. pylori* and to assess whether the administration of higher doses of esomeprazole could lead to improved eradication rates.

METHODS

We prospectively recruited in this pilot study 156 consecutive *H. pylori* positive outpatients, complaining of dyspeptic symptoms, who were referred to our department for upper gastrointestinal endoscopy for the first time. The demographic and endoscopic data of these patients are reported in Table 1. Previous *H. pylori* eradication therapy was an exclusion criterion for the study. We also excluded from the study patients with liver cirrhosis, renal failure, other serious concomitant illness, alcoholics; patients who had been treated in the preceding 2 months with antibiotics, bismuth preparations, proton pump inhibitors; patients with known allergy to the medications used; patients with a history of previous gastric surgery, and pregnant women. These criteria were ascertained by taking a complete history, physical examination, and appropriate hematologic and biochemical tests. The study was approved

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From the Gastroenterology Department, 251 Hellenic Air Force and Veterans General Hospital, Athens, Greece

Reprints: Dr. George K. Anagnostopoulos, 34 Dimokritou str, Agia Paraskevi, 15343, Athens, Greece (e-mail: gkanagnostopoulos@yahoo.gr).

TABLE 1. Demographic Data and Endoscopic Appearance of the Two Study Groups

	EAC1 Group	OAC Group	EAC2 Group
No. of patients	52	52	52
Men/Women	24,28	27/25	28/24
Age (mean ± SD)	55 ± 21	59 ± 19	57 ± 20
Range	27–81	24–80	19–78
Smokers	11 (21%)	13 (25%)	10 (19%)
Endoscopic diagnosis			
Gastritis	29 (56%)	28 (54%)	27 (52%)
Duodenitis	11 (21%)	9 (17%)	10 (19%)
Duodenal ulcer	3 (6%)	4 (8%)	5 (10%)
Reflux esophagitis	9 (17%)	9 (17%)	8 (15%)
Gastric ulcer		2 (4%)	2 (4%)

EAC, indicates esomeprazole-amoxycillin-clarithromycin; OAC, omeprazole-amoxycillin-clarithromycin.

by the Ethics Committee of our hospital, and informed written consent was obtained from all patients prior to the study.

During endoscopy 6 biopsy specimens (3 from the corpus, and 3 from the antrum) were taken. Two biopsy specimens (1 each from the antrum and corpus) were used for rapid urease test (CLO test, Ballard Medical Products, UT), and the rest of the specimens were sent for histologic assessment of *H. pylori* status by hematoxylin and eosin and Giemsa stain. *H. pylori* infection was defined when positive results in both the rapid urease test and histology were obtained.

The enrolled patients were randomized by drawing a sealed envelope that contained pre-assigned treatment instructions. They were allocated to receive 1 of the following treatments: (1) EAC1 group: 1-week treatment including esomeprazole 40 mg once daily, amoxicillin 1 g, and clarithromycin 500 mg, both twice daily; (2) OAC group: 1-week treatment of omeprazole 20 mg twice daily, amoxicillin 1 g, and clarithromycin 500 mg, both twice daily, or (3) EAC2 group: 1-week treatment with esomeprazole 40 mg, amoxicillin 1 g, and clarithromycin 500 mg, all given twice daily. Patients with duodenal ulcers in the OAC group were also given omeprazole (20 mg/d) for a total of 1 month. Both esomeprazole and omeprazole were administered 1 hour before breakfast and dinner. Patients, who had difficulty swallowing the tablet, were allowed to mix it with apple juice.

Each patient was asked to return at the end of the antibiotic treatment of a personal interview to assess any adverse events and compliance. Compliance was checked by counting unused returned study medication at the completion of treatment, and was considered excellent if the patient used 100% of the medication, good if the patient used >90% of the medication, and fair if the patient used <90% of the medication.

Five to 6 weeks after concluding proton pump inhibitor therapy *H. pylori* eradication was assessed by endoscopy, as at entry, and ^{13}C-Urea breath test (Helicobacter test; INFAI UK Ltd., Hensington, York, U.K.). Eradication was defined as negative result from both histologic assessment and urea breath test.

The results of the treatment were evaluated with per protocol analysis (which considers only the patients concluding the study) and the intention-to-treat analysis (which includes also the patients who dropped out).

RESULTS

H. pylori was eradicated in 37 of 52 patients in the OAC group (ITT 71%, 95% CI: 65%–77%), in 42 of 52 patients in the EAC1 group (ITT 81%, 95% CI: 75% 86%), and in 50 of 52 patients in the EAC2 group (ITT 96%, 95% CI: 91%–99%). Thus, OAC, and EAC1 groups gave comparable results with overlapping 95% CI, while EAC2 group had non-overlapping 95% CI.

All patients completed treatment in the OAC, and EAC1 groups. On the EAC2 group, 1 patient stopped treatment because of severe abdominal pain and was considered a dropout (Table 2).

In 3 patients in the EAC1 group, and in 5 patients in the EAC2 group the endoscopic diagnosis was duodenal ulcer. Endoscopic examination at the end of the study demonstrated that all ulcers were healed.

Side effects (headache, diarrhea, abdominal pain, flatulence, and nausea) were noticed in 8 patients in the OAC group (15%), 9 patients in the EAC1 group (17%). In both groups, side effects were mild, and did not necessitate any additional treatment. In the EAC2 group, 24 of 52 (46%) patients developed side effects (Table 3). Although in the majority of patients the side effects were mild, 1 patient stopped treatment due to abdominal pain, as already mentioned.

DISCUSSION

Esomeprazole, the *S*-isomer of omeprazole (a racemic mixture of *S*- and *R*- optical isomers), is the first proton pump

TABLE 2. Results of Treatment

	OAC Group	EAC1 Group	EAC2 Group
No. of patients	52	52	52
Drop-out	0	0	1
Cure rates			
ITT (n)	71% (37/52)	81% (42/52)	96% (50/52)
95% CI	65–77%	75–86%	91–99%
PP (n)	71% (37/52)	81% (42/52)	98% (51/52)
95% CI	65–77%	75–86%	92–99%

ITT, indicates intention to treat; PP, per protocol analysis

TABLE 3. Side-Effects During Treatment

	OAC Group	EAC1 Group	EAC2 Group
Headache	3	4	8
Abdominal pain	1	1	4
Diarrhea	3	1	3
Nausea	1	2	4
Flatulence	0	1	5
Total	8/52 (15%)	9/52 (17%)	24/52 (46%)

inhibitor (PPI) developed as a single optical isomer. Although esomeprazole is an optical isomer of omeprazole, it has different pharmacodynamic and pharmacokinetic profiles.[6,8]

In vitro studies indicate that esomeprazole is primarily metabolized via CYP2C19, which forms inactive metabolites.[9–12] Esomeprazole has minimal first pass metabolism, undergoes less hydroxylation via CYP2C19, and has lower intrinsic clearance than the R-isomer of omeprazole.[13] The end result in humans, given comparable doses, is for esomeprazole to produce an area under the plasma concentration curve (AUC) for the active substance that is 3 to 4 times greater than the R-omeprazole.[11] Potentially, higher AUC values lead to increased delivery of the drug to the canalicular lumen of the parietal cell and hence more pronounced inhibition of acid secretion. It is also known that the CYP2C19 isoenzyme exhibits genetic polymorphism, where approximately 3% of white and 15% to 20% of Asians are poor metabolizers[1,4] Because esomeprazole metabolism is affected to a much lesser extent, there is less interpatient variability between poor and extensive metabolizers of CYP2C19.[12] Thus, the higher and more consistent bioavailability of esomeprazole provides the rationale for its superior control of gastric acid secretion and improved clinical efficacy compared with omeprazole.

Several pharmacokinetic studies have compared the ability of esomeprazole to achieve acid control with currently available agents.[8,15–25] Esomeprazole 20 mg and 40 mg once daily for 5 days maintained intragastric PH greater than 4 for a greater length of time when compared with omeprazole 20 mg once daily in 36 patients with symptoms of gastroesophageal reflux disease (GERD).[8] Once daily administration of esomeprazole 40 mg also increased intragastric PH to greater than 4 for a longer period than omeprazole 40 mg once daily in 130 patients with GERD.[18] It is well known that the reduction of gastric acid secretion leads to increased availability and activity of some antibiotics Thus, the greater gastric acid inhibition seen with esomeprazole, could lead to better eradication rates of *H. pylori*, when used in triple therapy regimens.

Besides their inhibitory effect on acid secretion, PPIs have also been shown to exert an antibacterial activity in vitro, which is selective to *H. pylori*. Recently, Gatta et al[7] studied 52 *H. pylori* isolates obtained from gastric biopsies. Sixty-three percent of the isolates showed the same degree of susceptibility to esomeprazole and omeprazole, whereas 32.7% were 2-to 64-fold more susceptible to esomeprazole. The MIC50 and MIC90 of esomeprazole were 16 mg/L and 32 mg/L; and those of omeprazole were 32 mg/L and 64 mg/L. That means that the increased antimicrobial activity in vitro of esomeprazole against *H. pylori* could contribute to improving the outcome of the eradication treatment of this infection.

Few controlled, prospective, randomized trials have been conducted with esomeprazole- based therapy (Table 4).[25–27] Tulassay et al[25] evaluated a 7-day esomeprazole-based triple therapy in patients with active duodenal ulcer. *H. pylori* eradication rates were comparable for omeprazole versus esomeprazole, as was ulcer healing. Similar results were demonstrated in the study by Van Zanten et al.[26] Ten days' esomeprazole monotherapy dual therapy and triple therapy were assessed in patients with active or prior duodenal ulcer disease in the study by Laine et al.[27] Esomeprazole-based 10-day triple therapy was superior to dual therapy and monotherapy as was expected. In the studies by Tulassay and Van Zanten, esomeprazole was administered at a dose of 20 mg twice daily, whereas in the study by Laine et al the used dose was 40 mg once daily.

In our study we compared two 7 days esomeprazole-based triple regimens with a 7-day omeprazole based triple

TABLE 4. Overview of All Trials Evaluating Esomeprazole-Based Triple Regimens for the Eradication of H. Pylori

	Patients	Duration (days)	Regimen	Eradication Rate
Laine et al	233	10	ESO 40 mg od + AMC 1 g bid + CLA 500 mg bid	ITT: 77%, PP: 84%
	215	10	ESO 40 mg od + CLA 500 mg bid	ITT: 52%, PP: 55%
Tulassay et al	214	7	ESO 20 mg bid + AMC 1 gr bid + CLA 500 mg bid	ITT: 86%, PP: 89%
	219	7	OMP 20 mg bid + AMC 1 gr bid + CLA 500 mg bid	ITT: 88%, PP: 90%
van Zanten et al	204	7	ESO 20 mg bid + AMC 1 gr bid + CLA 500 mg bid	ITT: 90%, PP: 91%
	196	7	OMP 20 mg bid + AMC 1 gr bid + CLA 500 mg bid	ITT: 88%, PP: 91%

ESO, esomeprazole; AMC, amoxicillin; CLA, clarithromycin; od, once daily; bid, twice daily

regimen. The eradication rates achieved with esomeprazole 40 mg once daily were satisfactory (ITT > 80%), and comparable with the rates achieved with the 7-days omeprazole regimen. The administration of esomeprazole at a dose of 40 mg twice daily resulted in excellent eradication rates (ITT: 96%, PP: 98%). Little information exists in the literature about acid inhibition with esomeprazole 80 mg, but preliminary results are very positive. It seems that 40 mg of esomeprazole twice daily is extremely effective in controlling gastric acidity.[28]

In all recent studies administering esomeprazole at a dose of 40 mg once daily, or 20 mg twice daily, the adverse event rates were similar to those recorded for omeprazole or placebo.[29,30] In our group, the daily administration of 80 mg of esomeprazole, resulted in an increase in unwanted effects, but they were generally mild and only 1 patient stopped treatment.

Several limitations exist in our study; antibiotic resistance was not assessed, there was no pre-determined sample size calculations, and the number of patients included was relatively small. Yet, our results suggest that high dose esomeprazole regimens give excellent eradication rates. One explanation for our results is that because esomeprazole has better antimicrobial activity, and greater acid inhibitory ability than omeprazole, higher dose of the drug may help in overcoming antibiotic resistance, and increase the availability and activity of several antibiotics against *H. pylori*.

In conclusion, our pilot study suggests that high dose esomeprazole regimens may prove to be very effective for the eradication of *H. pylori*. Further properly powered studies, including higher numbers of patients, should be conducted to assess the efficacy and tolerability of high-dose esomeprazole eradication regimens.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-8538 1893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• *H pylori* •

Impact of *Helicobacter pylori* infection on histological changes in non-erosive reflux disease

Anthie Gatopoulou, Konstantinos Mimidis, Alexandra Glatromanolaki, Alexandros Polichronidis, Nikolaos Lirantzopoulos, Efthimios Sivridis, George Minopoulos

Anthie Gatopoulou, Konstantinos Mimidis, George Minopoulos, Endoscopy Unit, Democritus University of Thrace, Alexandroupolis, Greece
Konstantinos Mimidis, First Department of Internal Medicine, Democritus University of Thrace, Alexandroupolis, Greece
Efthimios Sivridis, Alexandra Glatromanolaki, Department of Pathology, Democritus University of Thrace, Alexandroupolis, Greece
Alexandros Polichronidis, Second Department of Surgery, Democritus University of Thrace, Alexandroupolis, Greece
Nikolaos Lirantzopoulos, George Minopoulos, First Department of Surgery, Democritus University of Thrace, Alexandroupolis, Greece
Correspondence to: Konstantinos Mimidis MD, Gastroenterologist, Lecturer in Internal Medicine, Chrisostomou Smirnis 8, 68100 Alexandroupolis, Greece. kmimidis@otenet.gr
Telephone: +3-2551074090 **Fax:** +3-2551030450
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Abstract

AIM: The evidence for an association between *Helicobacter pylori* (*H pylori*) and gastroesophageal reflux disease, either in non- erosive (NERD) or erosive esophagitis (ERD) remains uncertain. The available data on the histological changes in NERD and the effect on *H pylori* infection on them are elusive. The aim of this study therefore was to prospectively evaluate the histological findings and the impact of *H pylori* infection on a group of symptomatic patients with NERD.

METHODS: Fifty consecutive patients were prospectively evaluated for symptoms compatible with GORD. In all cases, routine endoscopy and lugol directed biopsies were performed and assessed histologically in a blinded manner.

RESULTS: The overall prevalence of *H pylori* infection was 70%. Twenty-nine patients out of 50 (58%) were NERD patients. No statistical significance was observed between the *H pylori* status and NERD. The remaining 21 (42%) were diagnosed as follows: 13 (26%), 6 (12%), 2(4%) with esophagitis grade A, B and C respectively. A statistically significant correlation was observed between the *H pylori*+ and esophagitis grade A, as well as between *H pylori*- and grade B. Biopsies from 2 patients were not included because of insufficient materials. Histologically, a basal zone hyperplasia was found in 47 (97.91%) patients, alterations of glycogen content in 47 (97.91%), papillae elongation in 33 (68.75%), blood vessels dilatation in 35(72.91%), chronic inflammation in 21 (43.75%), infiltration with eosinophils, neutophils and T-lymphocytes in 4 (8.33%), 6 (12.5%) and 39 (81.25%) respectively. No correlation was observed between the *H pylori* status and the histological parameters studied either in NERD or GERD.

CONCLUSION: Histological assessment can not differentiate symptomatic patients with erosive versus non-erosive reflux disease. Moreover, *H pylori* infection may not act as an important factor in patients with NERD.

Gatopoulou A, Mimidis K, Glatromanolaki A, Polichronidis A, Lirantzopoulos N, Sivridis E, Minopoulos G. Impact of *Helicobacter pylori* infection on histological changes in non-erosive reflux disease. *World J Gastroenterol* 2004; 10(8): 1180-1182
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INTRODUCTION

Helicobacter pylori (*H pylori*) is a prevalent pathogenetic factor associated with ulceration, dyspepsia, and adenocarcinoma[1,2]. The role of *H pylori* in gastroesophageal reflux disease (GORD) has only recently received attention, with the evidence for an association between *H pylori* and GORD remaining uncertain[3]. Diminution of peptic ulcer disease and adenocarcinoma of the distal stomach have paralleled the decreasing prevalence of *H pylori* infections in the developed world. At the same time, there has been an increase in GORD, Barrett' s esophagus, and adenocarcinoma of the distal esophagus and proximal stomach, suggesting that *H pylori* protects against these esophageal diseases[4,5].

Adequate assessment of reflux esophagitis has proved difficult to be assessed by endoscopy only, as the endoscopic appearance of the esophageal mucosa may be normal despite the presence of reflux symptoms[6,7]. Non-erosive gastroesophageal reflux disease (NERD) is the most common diagnosis in patients with reflux symptoms when organic diseases such as ulcers, esophageal erosions, and carcinomas, have been excluded by esophagogastroduodenoscopy[8].

Histological abnormalities have been described in GORD[9,10], and hence it seems reasonable to diagnose non-erosive reflux disease by simple esophageal biopsies during endoscopy. These mild histological findings are mainly basal zone thickening, elongated papillae, alterations in intracellular glycogen content, infiltration with neutrophils, eosinophils and T-lymphocytes, and submucosal blood vessel dilatation[11,12]. However, the available data on the diagnostic value of these histological criteria are contradictory[13]. This study therefore evaluated prospectively the histological findings and the impact of *H pylori* infection in a group of symptomatic patients with erosive and non- erosive reflux disease.

MATERIALS AND METHODS

Patients

Fifty patients (29 men, 21 women; mean age 49.9 years) were evaluated prospectively in our endoscopic unit for symptoms compatible with GORD, namely heartburn, acid regurgitation, and/or epigastric pain. A standardized questionnaire was completed for each patient during an interview with an experienced gastroenterologist. Demographic details of the GORD patients were recorded, including age, sex, smoking and drinking habits, tea and coffee consumption, and concurrent medical conditions including hypertension and diabetes mellitus. None of the patients included in this study had a

current or past history of peptic ulcer disease, previous gastric surgery or anti-*Helicobacter* therapy, or use of proton pump inhibitors, NSAIDs, steroids, or tetracycline during the past 4 wk. Ethics approval was obtained from the Ethics Committee of the University Hospital of Alexandroupolis, and patients gave their informed signed consent for biopsy specimens to be taken.

Methods

A routine endoscopy was performed by the same endoscopist on all patients using an (GIF-Q145) Olympus flexible endoscope. The distance between the esophagogastric junction and the incisor teeth was recorded. Reflux esophagitis was graded in accordance with the Los Angeles classification[7,8]. *H pylori* status was determined by the rapid urease test and histological examination of biopsies taken from the antrum and the corpus[9,10].

At least 4 biopsy specimens were taken 3 cm above the lower esophageal sphincter with Olympus biopsy forceps in a cross-fashion manner. In order to improve endoscopic visualization and provide biopsy orientation, 20 mL of 20 mg/L potassium iodine' s solution (Lugol) was applied through a "spray" catheter[10-12]. To obtain sufficient material and to ensure an almost vertical pinch biopsy specimen, the opened forceps were withdrawn towards the tip of the scope, which was bent towards maximally, and hence the forceps were pressed vertically against the esophageal wall. Specimens were fixed in 40 g/L formaldehyde[13]. When all sections had been selected they were assessed histologically in a blinded manner (without endoscopic or clinical information). A standardized report completed by the histopathologist comprised an evaluation of the following histological parameters: basal zone hyperplasia, papillary length, dilatation of intraepithelial blood vessels, and semiquantitative cellular infiltration with T-lymphocytes, neutrophils, eosinophils. Alterations of glycogen content, erosion, ulceration and chronic inflammation were also assessed[9,10,11].

Statistical analysis

Statistical analysis was performed using SPSS (version 11.0 for Windows) on data from all 50 patients. The analysis was based on demographic characteristics, such as age, sex, and presence of *H pylori* infection, as well as endoscopic and histological findings. Differences in the distribution of the variables of interest between subgroups of patients were examined by Pearson chi-square test or Fisher's exact test (the latter when small frequencies were present). Comparison between proportions in 2 independent groups was performed with the z-test statistic. *P*-values less than 0.05 were considered significant.

RESULTS

The relationship between endoscopic findings and the presence (*H pylori*+) or absence (*H pylori*-) of *H pylori* infection is shown in Figure 1.

The overall prevalence of *H pylori*+ was 70% (35 out of 50 patients). A normal appearance of esophageal mucosa (non-erosive esophagitis) was observed in 29 out of 50 (58%) patients. No statistical significance was observed between *H pylori*+ and NERD patients [*H pylori* + in 20 out of 35 (57.1%) *vs H pylori* - in 9 out of 15 (60%) patients *P*>0.05]. The remaining 21 (42%) patients with erosive esophagitis were diagnosed as follows: 13 (26%), 6 (12%) and 2 (4%) with esophagitis grades A, B and C respectively. None of the patients in our series suffered from esophagitis grade D. A statistically significant correlation was observed between the *H pylori*+ and esophagitis grade A [*H pylori*+ in 11 out of 35 (31.4%) *vs H pylori*- in 2 out of 15 (13.3%) patients, *P*<0.05]. Similarly, a statistical difference was observed in the group of patients with esophagitis grade B [*H pylori*+ in 2 out of 35 (5.7%) *vs H pylori*- in 4 out of 15 (26.7%) patients, *P*<0.05].

Figure 2 summarizes the distribution of patients according to biopsy findings and the existence of *H pylori*. Biopsies from 2 patients were not included because of insufficient materials. No difference was observed between the 2 variables of interest. As expected, the majority of patients examined (46 of 48, 95.8%) were diagnosed histologically as having esophagitis, despite the esophageal mucosa appearing normal under endoscopy.

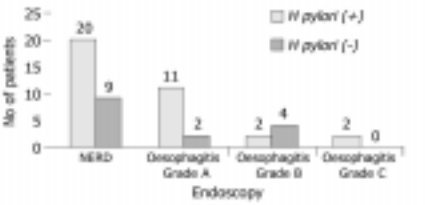

Figure 1 Findings at endoscopy. Note: *H pylori* (+): *Helicobacter pylori* positive patients; *H pylori* (-): *Helicobacter pylori* negative patients.

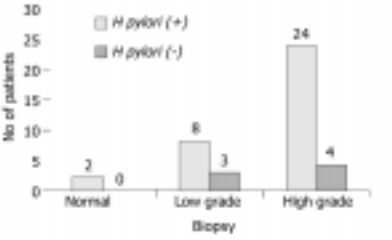

Figure 2 Findings at biopsy. *H pylori* (+): *Helicobacter pylori* positive patients, *H pylori* (-): *Helicobacter pylori* negative patients.

Table 1 Correlation of *H pylori* infection with the histological parameters esophagitis in NERD and GERD

Histological parameters	Total (*n*) of patients	NERD		ERD		*P*
		Patients (*n*) (%)	*H pylori* (+) (%)	Patients (*n*) (%)	*H pylori* (+) (%)	
Basal zone hyperplasia	47	28 (59.6)	19 (67.9)	19 (40.4)	14 (73.7)	0.462
Loss of glycogen	47	28 (59.6)	19 (67.9)	19 (40.4)	14 (73.7)	0.462
Papillae elongation	33	18 (54.4)	11 (61.1)	15 (45.6)	11 (73.3)	0.337
Blood vessels dilatation	35	20 (57.1)	15 (75.0)	15 (42.9)	12 (80.0)	0.527
Eosinophils infiltration	4	2 (50.0)	1 (50.0)	2 (50.0)	2 (100.0)	0.500
Neutrophils infiltration	6	2 (33.3)	1 (50.0)	4 (66.7)	3 (75.0)	0.600
T-lymphocytes infiltration	39	24 (61.5)	17 (70.8)	15 (38.5)	11 (73.3)	0.582
Chronic inflammation	21	13 (61.9)	10 (76.9)	8 (38.1)	5 (62.5)	0.410

Finally, Table 1 outlines the histological parameters of esophagitis with the presence or absence of erosions during endoscopy. We focused on patients with *H pylori* infection. No statistically significant difference was observed between *H pylori* (+) patients with erosive (ERD) and non-erosive esophagitis (NERD) for any of the histological parameters examined (*P* values refer to the numbers in italics).

DISCUSSION

This study investigated the impact of *H pylori* infection on the histological changes of non-erosive esophagitis. Our study was performed prospectively in a series of 50 patients with reflux symptoms. Esophageal erosions were found during endoscopy in 21 of these patients. The overall prevalence of *H pylori*+ was 70% (erosive: 15/21, 71.5%; non-erosive: 20/29, 68.9%). As expected, histological changes were noted in the majority of biopsies (95.8%) despite a normal appearance of esophageal mucosa under endoscopy in half of the cases.

In the group of patients with erosive esophagitis, *H pylori*+ was correlated with grade A esophagitis, whereas *H pylori*- was correlated with grade B disease. This probably indicates that *H pylori*- is a risk factor for ERD and aggravates the endoscopic appearance. This finding of a close association between *H pylori*+ and less serious endoscopic findings is consistent with other studies[14,19-21]. Some studies have found an inverse relation between *H pylori* and esophagitis, once *H pylori* has been eradicated[22,23]. In our series no patient had previously received eradication therapy. Furthermore, we confirmed previous findings that *H pylori* infection was not associated with positive or negative esophagitis findings in biopsies[24,25].

In the second group of patients (those with non-erosive esophagitis), no correlation was observed between the *H pylori*+ and the histological parameters studied. Little is known about the relationship between *H pylori* infection and the histological variables in non-erosive esophagitis. The fact that there appears to be no correlation between the *H pylori*+ and any of the aforementioned mild changes probably implies that these are provoked by mechanisms other than *H pylori* infection[25-27] (perhaps acid or bile reflux).

In conclusion, *H pylori* probably plays a statistically unimportant role in the histological changes seen in patients with non-erosive reflux disease, since similar histological alterations were detected in biopsies of both erosive and non-erosive esophagitis. Further research is required to identify other pathogenetic factors responsible for the histological parameters found in this group of patients.

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GASTROENTEROLOGY

Relationship of smoking and coffee and alcohol consumption with seroconversion to *Helicobacter pylori*: A longitudinal study in hospital workers

ARISTOFANIS GIKAS,* JOHN K TRIANTAFILLIDIS,* NIKOLAOS APOSTOLIDIS,† ELIAS MALLAS,‡ GEORGE PEROS§ AND GEORGE ANDROULAKIS†

**Department of Gastroenterology, Saint Panteleimon General State Hospital, Nicea, †First Department of Surgery, Hippokration Hospital, University of Athens, ‡Department of Gastroenterology, Areteio Hospital and §Fourth Department of Surgery, University of Athens, Athens, Greece*

Abstract

Background and Aim: There are few data concerning the relationship between *Helicobacter pylori* seroconversion, and smoking habits and coffee and alcohol consumption. The aim of the present study was to investigate the relationship between smoking habits, coffee and alcohol consumption, and *H. pylori* seroconversion.

Methods: The data used were derived from a sample of 238 subjects (hospital employees) who were initially (on 1994) seronegative to *H. pylori*. These subjects were tested again 5 years later (1999). Information concerning smoking habits and coffee and alcohol consumption was collected by the use of a special questionnaire, which was completed by the same cohort of subjects in two different periods (1994 and 1999). Logistic regression was used to assess the relationship between *H. pylori* seroconversion and the aforementioned lifestyle factors.

Results: Neither smoking nor coffee consumption was significantly related to *H. pylori* seroconversion. Adjusted odds ratio for alcohol drinkers as compared with non-/occasional drinkers was 0.59 (95% confidence interval [CI]: 0.31–1.16, $P = 0.13$). However, the odds ratio was significantly lower (0.26, 95%CI: 0.07–0.95, $P = 0.042$) in subjects who reported moderate alcohol consumption at first (1994) examination, as compared with non-/occasional drinkers. Small and heavy drinking were not associated with *H. pylori* seroconversion.

Conclusions: There is no significant relation between *H. pylori* seroconversion and smoking and coffee consumption. The present findings suggest that moderate alcohol consumption might be inversely associated with *H. pylori* seroconversion.

Key words: alcohol, coffee, *Helicobacter pylori*, seroconversion, smoking.

INTRODUCTION

Helicobacter pylori is a common bacterium affecting almost one-half of the world population. It has long been recognized as a major cause of gastritis, peptic ulcer, gastric lymphoma and gastric cancer.[1–3] During the last decade much has been learned concerning different biological and clinical aspects of *H. pylori* infection, but the precise mode of transmission has not yet been determined. In an attempt to clarify this issue, epidemiological studies have found that age, race and education level (as a strong indicator of socioeconomic status) were major predictive factors of infection.[4,5]

So far, several studies have tried to assess the possible association between *H. pylori* infection and smoking habits and coffee and alcohol consumption.[5–23] These studies were cross-sectional prevalence studies and gave inconsistent and sometimes conflicting results.

The objective of the present study was to investigate the relationship between *H. pylori* seroconversion

Correspondence: Dr A Gikas, 6 Gavrilidou Street, 11141 Ano Patisia, Athens, Greece. Email: argikas@internet.gr
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(change from negative to positive status for antibodies against *H. pylori*) and smoking habits and coffee and alcohol consumption in a cohort of hospital workers of a large acute care hospital in Greece at two different time periods (first trimester of the years 1994 and 1999). The study was approved by the ethics committee of Saint Panteleimon General State Hospital.

METHODS

Subjects

Two hundred and thirty-eight hospital workers out of 437 (ages 23–55 years, mean age: 34 ± 6.7 years) who were negative for the presence of *H. pylori* serum antibodies at the initial examination (1994), were the object of the present study. This cohort of individuals was tested again in 1999. Other subject data are shown in Table 1.

Data collection

A self-report questionnaire was completed by all subjects at the same time as their blood specimens were taken. Special effort was taken to obtain and record details concerning smoking habits and coffee and alcohol consumption. In particular, with respect to smoking, the questionnaire asked whether the subject smoked cigarettes currently, in the past, or never. Smokers were asked to indicate how many cigarettes per day they smoked.

The smoking habit, which represented a qualitative aspect of smoking, was categorized into current smokers and-non-smokers (including those who gave up smoking). Cigarette consumption was expressed as cigarettes smoked per day and divided into different categories: 0, 1–14, 15–24, and ≥25.

Table 1 Subject data

Parameter	*n*	%
Age (years)		
23–34	118	49.6
35–55	120	50.4
Sex		
Female	179	75.2
Male	59	24.8
Educational level		
Elementary	33	13.9
Moderate	111	46.6
Higher	94	39.5
Profession		
Administrative and technical staff	71	29.8
Nursing staff	128	53.8
Paramedical staff	21	8.8
Medical staff	18	7.6

As far as coffee consumption was concerned, subjects were divided in categories according to the number of coffee cups consumed per day (0, 1–2, ≥3).

With respect to alcohol consumption, the questionnaire asked whether the subject drank or not. The current drinkers were asked to record the amount of wine, beer and/or whisky consumed per day, week or month. Based on these data the amount of pure alcohol consumed per week was calculated assuming that, in Greece, 1 L of wine or beer contained on average 100 g or 40 g ethanol, respectively. According to the amount of pure ethanol consumed per week, subjects were divided into non- or occasional drinkers (i.e. abstainers and subjects who drank on the occasion of a social event, e.g. less than once a week or less than four times per month; these subjects were used as the referent group) and drinkers (i.e. subjects who consumed <70 g, 70–140 g or >140 g per week).[22] They were also divided into different categories according to the kind of beverage (only wine; only beer; or mixed).

The same questionnaire was completed by the same cohort of subjects at both time periods (1994 and 1999).

Measurement of serum antibodies against *H. pylori*

Serum antibodies against *H. pylori* were measured using an ELISA kit (Hycor Biomedical, Kassel, Germany). The test was assigned as positive if the value of IgG antibodies in the serum was >40 U/mL. Although the sensitivity and specificity of the immunoassay were greater than 95% and 96%, respectively, according to the manufacturer, the two parameters were again validated at Saint Panteleimon General State Hospital and were found to be greater than 96%. The test for the detection of *H. pylori* infection in both periods of time was the same. Tests were performed by the same laboratory staff and under the same conditions.

Statistical analyses

The possible association of any of lifestyle factors that we studied (smoking, coffee and alcohol consumption) with the seroconversion was initially analyzed using univariate analysis (χ^2 test and *t*-tests). After that, different models of multivariate logistic regression analysis were applied, controlling for all available covariate variables that were known or suspected to be related with *H. pylori* infection. The conditions for the application of the logistic regression analysis were checked and the existence of interaction was studied. We finally calculated in each group the odds ratio (OR) of seroconversion adjusted for age, sex, educational level, profession, and for each other of the lifestyle habits. Adjusted OR of *H. pylori* seroconversion was used as the measure of association, and these were presented together with 95% confidence intervals (CI). Effects were considered to be statistically significant if the 95% CI did not

include the value 1.0, corresponding with two-sided $P < 0.05$.

RESULTS

During the 5-year observation 59 out of 238 (24.8%) subjects initially negative for *H. pylori* infection became positive, thus giving an annual seroconversion rate of approximately 4.95%. The multivariate analysis showed that, among covariate variables, age and nursing profession were independent risk factors for seroconversion. Details concerning the relationship between *H. pylori* infection with other clinicoepidemiological parameters in the whole cohort of subjects (n = 437), have previously been published elsewhere.[24]

Smoking habit

At the initial phase of the study (1994), the proportion of smokers who became positive for *H. pylori* antibodies, was not different from that of non-smokers (Table 2). No relationship between the number of cigarettes smoked per day and seroconversion was found. During the follow-up period 26 individuals changed their smoking habits. After exclusion of these subjects from the analysis the percentage of seroconversion in persistent smokers and non-smokers did not change (25.2% *vs* 24.8%, $P = 0.93$). Again no significant difference in the percentage of seroconversion was found in the four categories based on the number of cigarettes smoked per day (25.2% *vs* 27.3% *vs* 26.7% *vs* 21.1%).

Coffee consumption

The vast majority of hospital workers (89.5%) consumed coffee daily. As shown in Table 2, the percentage of seroconversion concerning the groups without coffee consumption and those groups with 1–2 and ≥3 cups per day did not differ significantly ($\chi^2 = 0.48$, $P = 0.79$). Although the percentage of seroconversion was greater in those subjects with heavy coffee consumption (≥3 cups/day), the adjusted OR was not significantly higher compared to subjects without coffee consumption (OR: 1.35, 95% CI: 0.38–4.85, $P = 0.64$).

Again no significant differences were found on 1999 analysis (19% *vs* 24.2% *vs* 28.3%) concerning the aforementioned parameters.

Alcohol consumption

On the initial examination (1994), 144 subjects (60.5%; 39.5% of men and 70.4% women), reported no or occasional alcohol consumption. From the remaining 94 subjects, 40 (42.5%) were mild consumers (<70 g of ethanol per week), 34 (36.2%) reported moderate consumption (70–140 g of ethanol per week) and 20 (21.3%) reported heavy alcohol consumption (>140 g). Thirty-one per cent of drinkers consumed only wine, 23% consumed only beer and 46% consumed both.

As shown in Table 3, although the proportion of seroconverters among drinkers was smaller compared with that of non-/occasional-drinkers (19.1% *vs* 28.5%), this difference did not reach statistical significance. Adjusted OR for seroconversion were significantly lower, only in the group of moderate consumption as compared with the referent group (OR: 0.26, 95% CI: 0.07–0.95, $P = 0.042$). In other words, non-/occasional drinkers had a 3.8-fold higher risk for *H. pylori* seroconversion compared to those with moderate consumption of alcohol. The odds of seroconversion tend to increase with high consumption (Table 3). It must be noted that subjects with heavy consumption were statistically significantly older (38.6 ± 8 years *vs* 34 ± 7 years, $P = 0.042$) and less educated ($\chi^2 = 12.9$,

Table 2 Relation of smoking and coffee consumption with *Helicobacter pylori* seroconversion

	Seroconverters n (%)	P (χ^2)	Adjusted OR OR (95% CI)
Smoking habit			
Non-smokers[†]	31/120 (25.8)	0.71	—
Smokers	28/118 (23.7)		0.89 (0.44–1.78)
No. cigarettes/day			
0[†]	31/120 (25.8)	0.98	—
<15	6/24 (25.0)		1.34 (0.43–4.13)
15–24	13/54 (24.1)		0.86 (0.37–2.03)
≥25	9/40 (22.5)		0.68 (0.24–1.90)
Coffee consumption (cups/day)			
None[†]	5/25 (20.0)	0.79	—
<3	38/154 (24.7)		1.16 (0.37–3.67)
≥3	16/59 (27.1)		1.35 (0.38–4.85)

[†]Referent group. Adjusted odds ratio (OR): adjusted for age, sex, educational level, profession, alcohol consumption, smoking and coffee consumption. CI, confidence interval.

Table 3 Relation of alcohol consumption to *Helicobacter pylori* seroconversion

	Seroconverters *n* (%)	*P* (χ^2)	Adjusted OR OR (95% CI)
Alcohol drinking			
Non-/occasional drinkers[1]	41/144 (28.5)	0.10	—
Drinkers	18/94 (19.1)		0.59 (0.31–1.16)
Alcohol consumption (g/ week)			
Non-/occasional drinkers[1]	41/144 (28.5)	0.11	—
<70 g	9/40 (22.5)		0.68 (0.29–1.62)
70–140 g	3/34 (8.8)		0.26 (0.07–0.95)
>140 g	6/20 (30.0)		1.09 (0.37–3.46)
Beverage			
Non-/occasional drinkers[1]	41/144 (28.5)	0.34	—
Wine only	4/29 (13.8)		0.43 (0.13–1.39)
Beer only	5/22 (22.7)		0.50 (0.16–1.54)
Mixed	9/43 (20.9)		0.75 (0.32–1.76)

[1]Referent group. Adjusted odds ratio (OR) adjusted for age, sex, educational level, profession, smoking and coffee consumption. CI, confidence interval.

$P = 0.002$) as compared with those of moderate alcohol consumption.

Among nursing staff (a high-risk professional group for seroconversion) the proportion of seroconverters in each alcohol consumption group was 31.2% (25/80) among non-/occasional-drinkers, 28% (7/25) among those with mild consumption, 13.3% (2/15) among those with moderate and 25% (2/8) in those with heavy alcohol consumption. The application of separated multivariate model for nursing staff showed that the odds of seroconversion in subjects with moderate alcohol consumption were 0.27 (95% CI: 0.08–0.96, $P = 0.043$) as compared with the referent group, indicating that moderate alcohol consumption could have a protective effect in this particular high-risk group for *H. pylori* infection.

As shown in Table 3, although the proportion of seroconversion was smaller in those who consumed only wine compared with subjects who consumed beer or other beverages, this difference was not statistically significant.

The analysis of alcohol consumption based on 1999 data showed that the rate of seroconversion in non-/occasional drinkers was 28.2% as compared with 19.4% of drinkers (no significant differences). The rate of seroconversion in the four categories of alcohol consumption was 28.3% for non-/occasional consumers, 25% for those with mild consumption, 10.3% for those with moderate consumption and 27.8% with those with heavy consumption. Those with moderate alcohol consumption differ almost significantly compared with those without or with occasional alcohol consumption (adjusted OR: 0.32, 95% CI: 0.10–0.99, $P = 0.049$).

Moreover, when the 13 subjects (5.5%) who belonged in different categories in the two examinations (subjects who reported different alcohol consumption in 1994 and 1999 questionnaire) were excluded from analysis, the percentage of seroconversion was again significantly smaller in those who reported moderate alcohol consumption as compared with non-/occasional drinkers (9.7% vs 28.6%, adjusted OR: 0.27, 95% CI: 0.08–0.96, $P = 0.044$).

DISCUSSION

Previous studies concerning the possible relation between *H. pylori* and smoking, coffee and alcohol consumption reported inconsistent to contradictory results.[9–23] To our knowledge, this is the first longitudinal study evaluating concurrently the relationship between acquisition of *H. pylori* and smoking, coffee and alcohol consumption.

The rate of annual seroconversion rate found in the present study (4.95%), although quite high, must not be considered as an unexpected one, and correlates well with that described in previous seroprevalence studies concerning blood donors and healthy Greek population.[5,23,24] In a recently published study, a very high seroconversion rate was reported: 14.8% among 142 young male navy recruits, 8 months after their induction.[25] So far, the few studies conducted in developed countries found a quite low annual seroconversion rate in the general population (<1.1%),[26–28] although other studies concerning hospital workers found a higher seroconversion rate (e.g. 2.6% in doctors[30] and 2.3% in trainee nurses[31]). It is worth noting that the high rate of seroconversion observed in the present cohort of subjects could be attributed not only to the nature of their work (hospital employees) but also to the fact that Saint Panteleimon General State Hospital serves an urban area in which most of the inhabitants are of low socioeconomical level.

Smoking

Several studies have assessed the possible association between *H. pylori* infection and smoking. In the majority

of previous cross-sectional studies no relation of cigarette smoking with *H. pylori* prevalence was found.[4–7,10,11,13,14,16,21,25] However, there were some data in favor of a positively[17–19] or negatively[8,20] related smoking habit to *H. pylori* seropositivity.

In the present study and in concordance with most of the previously published studies, no relationship between smoking habits and seroconversion was found, indicating no direct effect of smoking on *H. pylori* infection. To explain the contradictory results described in previous studies, we suggest that these may be due to hidden confounding factors, mainly those related to socioeconomic class.

Coffee consumption

In a cohort study (using epidemiologists as the subjects) it was found that the risk of seroconversion was higher among those who drank more than 2 cups of caffeinated drinks per day than among the others.[14] Another, cross-sectional study reported that drinking coffee was associated with an increased prevalence of *H. pylori* infection.[7] On the contrary, other studies found no significant relation between coffee consumption and *H. pylori* infection.[9,11,14,23] The results of the present study are consistent with the latter, suggesting that no direct relationship between coffee consumption and *H. pylori* infection exists.

Alcohol consumption

Several cross-sectional studies have investigated the relationship between alcohol consumption and *H. pylori* infection. Some studies reported a significantly inverse association with *H. pylori* infection,[7,8,10,12,16,21] while others found no significant association.[5,6,9,11,13,16,17,19,20] In the present study we found that subjects with moderate alcohol consumption exhibited a marginally significantly lower risk for acquisition of *H. pylori* infection as compared with non-/occasional drinkers. The risk of seroconversion did not differ significantly in those subjects with small or heavy alcohol consumption as compared with non-/occasional drinkers. Similar findings to our results were reported by Brenner *et al.* and, recently, by Murray *et al.*, who found that moderate alcohol consumption might be protective against *H. pylori* infection.[12,23]

Our results also support the hypothesis that the association between alcohol consumption and *H. pylori* infection is not monotonic but U-shaped, due to the inverse association of *H. pylori* acquisition with moderate alcohol consumption. This finding is of some interest because a similar relationship (U-shaped) has been described between alcohol consumption and coronary heart disease,[33] which has been related to *H. pylori* infection in some studies.[34]

One of the possible explanations for the protective effect of alcohol against *H. pylori* acquisition is its direct antimicrobial effect against *H. pylori*.[35–37] It could be argued that moderate consumption of alcohol (e.g. 70–140 g/week, on average) could not allow the colonization of gastric mucosa by *H. pylori*, although it is not clear why heavy alcohol consumption does not exhibit such a protective effect. Indeed, some authors claimed that heavy alcohol consumption favors colonization of the gastric mucosa by *H. pylori*.[38] We suggest that other risk factors, mainly those related to unhealthy lifestyles, could be stronger than the effect of alcohol consumption. In favor of the latter is the fact that in the present study subjects reporting heavy alcohol consumption were both less educated and older, as compared with those reporting moderate alcohol consumption.

The findings of the present study could also explain differences observed in previously published cross-sectional studies. In our opinion, differences observed in previous studies are referring to the following points: (i) the use of dichotomous parameters concerning alcohol consumption, thus leading to loss of category of moderate consumption; (ii) the lack of control for confounding factors; and (iii) the cross-sectional design that did not take into account the real differences in drinking habits before the examination.

We must note, however, that the present study has some limitations. For example, the sample of subjects examined in both periods was relatively small and this may explain the marginally significant results (P: 0.042–0.049) that are in some way of limited power. Also, the fact that the majority of the subjects were women, influenced the number of subjects in each category of alcohol consumption, thus explaining in part the relatively high proportion of non-/occasional drinkers. We must emphasize, however, that the proportion of non-/occasional drinkers and other categories of drinkers reported in the present study (separately for men and women) is not different to that described in the general Greek population.[39–41]

In conclusion the present study attempted to elucidate some aspects of the relationship of *H. pylori* infection with three of the most important lifestyle factors, namely alcohol and coffee consumption and smoking habits. It was found that there is no significant association between *H. pylori* seroconversion and smoking and coffee consumption. However, there were some indications suggesting that moderate alcohol consumption might be inversely associated with *H. pylori* seroconversion. Whether our results, referring to a group of hospital employees, could be universally applied, needs further investigation.

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Apoptosis and autoimmunity as proposed pathogenetic links between *Helicobacter pylori* infection and idiopathic achalasia

Jannis Kountouras*, Christos Zavos, Dimitrios Chatzopoulos

Department of Gastroenterology, 2nd Medical Clinic, Ippokration Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece

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Summary Achalasia is a disorder of the oesophagus characterised by increased lower oesophageal sphincter (LOS) tone, lack of LOS relaxation with swallowing and aperistalsis of the body of the oesophagus. The aetiology and pathogenesis of idiopathic achalasia are still unclear, although a viral cause, genetic influences (associations with HLA loci) and autoimmune processes have been postulated. Degeneration and significant loss of nerve fibres, associated with an inflammatory infiltrate of the myenteric plexus in idiopathic achalasia, provide evidence of an immune mediated destruction of the myenteric plexus, possibly through apoptotic process. This concept is reinforced by the concomitant appearance of achalasia and Guillain-Barré syndrome (GBS) and/or Parkinson's disease, where inappropriate initiation of apoptosis has been proposed to underlie the neuronal attrition. In the same respect, *Helicobacter pylori* (*H. pylori*) infection has been associated with gastric autoimmunity, and patients infected with *H. pylori* have been shown to possess autoantibodies that cross-react with antigens expressed on the gastric mucosa. Furthermore, *H. pylori* is thought to be associated with the development of autoimmune sequelae observed in peripheral neuropathies and GBS, where autoantibodies to specific neural targets have been found to impair native neural function by inducing nerve tissue damage, possibly by apoptosis. Taken together, we assume that *H. pylori* infection might be a pathogenetic factor of achalasia through induction of autoimmunity and apoptosis. Whether eradication of *H. pylori* infection may indirectly offer benefit to the pathophysiology of idiopathic achalasia by ameliorating the apoptotic loss of ganglion cells and their axons in the oesophageal wall remains to be elucidated.

Introduction

Achalasia is a disorder of the oesophagus characterised by increased lower oesophageal sphincter (LOS) tone, lack of LOS relaxation with swallowing and aperistalsis of the body of the oesophagus [1]. This disease is observed in men and women mainly in the fifth and sixth decades of life, although it can also be found at any age [2]. The aetiology and pathogenesis of idiopathic achalasia are still unclear. Although a viral cause has been postulated, electron microscopy of the vagus nerve and intramural plexus has not revealed viral particles, viral genomic products are not consistently recognised in the plexuses, and epidemiologic features do not

*Corresponding author. Present address: 8 Fanariou St, Byzantio 55133, Macedonia, Thessaloniki, Greece; tel.: +30-2310-892238/438714; fax: +30-2310-992794.
E-mail address: jannis@med.auth.gr (J. Kountouras).

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suggest an infectious cause [3]. Genetic influences also appear to have restricted contribution unless achalasia is related to a multisystem disorder, i.e., keratoconjunctivitis sicca linked with achalasia of the cardia, adrenocortical insufficiency and lacrimal gland degeneration, although associations with HLA loci have been described [4]. Data indicate that antibodies to myenteric neurones are present in 64% of patients with achalasia, which raises the possibility of an autoimmune process [5]. In particular, degeneration and significant loss of nerve fibres, associated with an inflammatory infiltrate [predominantly T lymphocytes (TLs)] of the myenteric plexus in idiopathic achalasia, provide evidence of an immune mediated destruction of the myenteric plexus [1,2], possibly through apoptotic process. This concept is reinforced by the concomitant appearance of achalasia and Guillain-Barré syndrome (GBS) and/or Parkinson's disease, where inappropriate initiation of apoptosis has been proposed to underlie the neuronal attrition [6–9].

In the same respect, *Helicobacter pylori* (*H. pylori*) – a curved spiral gram-negative bacterium that colonises the human stomach – prevalence increases with age. Moreover, the association between *H. pylori* infection and gastric autoimmunity is well established [10] and patients infected with *H. pylori* have been shown to possess autoantibodies that cross-react with antigens expressed on the gastric mucosa [11,12]. *H. pylori* appears to upregulate the expression of HLA class II (HLA-DR) molecules on gastric epithelial cells and induces their apoptosis [13,14]. Furthermore, *H. pylori* is thought to be associated with the development of autoimmune sequelae observed in peripheral neuropathies [15,16] and GBS [17], where autoantibodies to specific neural targets have been found to impair native neural function by inducing nerve tissue damage possibly by apoptosis.

Taken together, we assume that *H. pylori* infection might be a pathogenetic factor of achalasia through induction of autoimmunity and apoptosis, a mechanism of cell death occurring in several important neurodegenerative and gastrointestinal diseases including Sjögren's syndrome, GBS, Parkinson's disease, Alzheimer's disease, glaucoma, retinitis pigmentosa, cataract formation, retinoblastoma, retinal ischaemia, diabetic retinopathy and *H. pylori*-induced upper gastrointestinal disorders [18].

Pathophysiology of achalasia

The enteric nervous system (ENS) can be considered as the third component of the autonomous nervous system. It is a vast network of neurones extensively dispersed throughout the gut. The ENS is a dominant regulator of gut function through the action of peptide and non-peptide neurotransmitters. The most intensively studied roles of the ENS have been the regulation of secretory processes, such as gastric acid secretion, and motility [19]. Disturbances in both nerve and muscle components can be detected in achalasia, although the neural lesion is thought to be of primary importance. Thorough examination of the intramural oesophageal nerve plexus has established reduction in numbers of NANC (non-adrenergic, non-cholinergic) inhibitory ganglion cells, apparently the most important pathophysiologic deficiency [20]. An activated TL infiltrate surrounds the remaining degenerating nerve fibres and loss of inhibitory ganglia extends into the proximal stomach [1,21], possibly suggesting an apoptotic attack of TLs toward nerve fibres and ganglia. Loss of myenteric ganglion cells and nitrergic inhibitory neurones extends to the stomach in some achalasia patients, and motor abnormalities of the small intestine, gallbladder, and sphincter of Oddi have also been observed in patients with achalasia [2]. Electron microscopic examination of the oesophageal vagal branches reveals degeneration of myelin sheaths and disruption of axonal membranes, the valerian degenerative changes characteristic of experimental nerve transaction. Degenerative changes, including fragmentation and dissolution of nuclear material, have also been reported in ganglia of the vagal dorsal motor nucleus [22], changes that have been observed during the apoptotic process [23,24]. Notably, the extra-oesophageal neuropathic changes have been demonstrated in only small number of achalasia patients. Nevertheless, lesions of the vagus nerve or its motor nuclei are plausible in this disease, in as much as bilateral lesions in the feline dorsal motor nuclei of the vagus can induce dysfunction similar to achalasia. The interrelationship of ganglion cell loss from the oesophageal wall and the vagal and brainstem lesions is not wholly understood. A disorder affecting both intrinsic and extrinsic sites could be operational; on the contrary, some degree of vagal degeneration could result secondarily from loss of ganglion cells. The lower oesophageal circular muscle is thickened, but muscular changes are thought to be secondary to underlying neuropathy [3].

Physiologic studies have established the occurrence of denervation of the smooth muscle segment of the oesophagus in patients with achalasia. First, muscle strips from the body of the oesophagus contract in response to direct stimulation

(acetylcholine) but not in response to ganglionic stimulation (nicotine). Similarly, strips from the region of the LOS do not relax in response to ganglionic stimulation in patients with achalasia, in contrast to normal controls. Second, exaggerated contractions in the oesophagus body and sphincter can be calculated when patients with achalasia are given parenteral injection of acetylcholine analogue acetyl-β-metacholine. This response seems to be indicative of denervation hypersensitivity. Third, cholecystokinin octapeptide (CCK-8) induces an unexpected enhancement of LOS pressure in patients with achalasia. This effect may signify loss of inhibitory neurones in the LOS region, in as much as these neurones normally produce the predominant response to CCK-8 stimulation. All these events are evidence of functional impairment of intramural ganglion cells in the oesophageal body and LOS region [3].

Anatomic and physiologic aspects are sufficient for explaining the manifestations of this disorder. A loss of ganglion cells in the region of LOS, particularly if the loss is predominantly of inhibitory neurones, would lead to an increased basal pressure and poor relaxation under normal circumstances. Vagal changes, whether primary or secondary, could also predominantly affect inhibitory stimulation of sphincter muscle, further compounding this problem. Ganglion cells degeneration in the oesophageal body itself would ultimately result in permanent aperistalsis and allow for dilatation of the oesophagus. The damage in the LOS region might be the earliest event, and the aperistalsis of some early non-dilated cases might be related to obstruction of oesophagus at the level of the sphincter. After reduction in LOS pressure by pneumatic dilation or myotomy in these patients, occasional aperistalsis may be observed [3].

Aetiology of idiopathic achalasia

Although the underlying cause of idiopathic achalasia is unknown, the diffuse neuronal effects found suggest a possible viral, bacterial or neurodegenerative mechanism. Using serological methods, a significant association between the HLA-DQ1 phenotype and idiopathic achalasia has been observed, suggesting a possible immunogenetic mechanism [25]. Further subtyping in white subjects revealed a significant association between idiopathic achalasia and the DQB1*0602 allele. A strong trend was also observed with the DRB1*15 allele. In the black population, there was no association between idiopathic achalasia and DQB1*0602 or DRB1*15, but a trend was found with DRB1*12 [25]. Interestingly, the TNFa11 allele and the DRB1*1501-DQA1*0102-DQB1*0602 haplotype is reduced in achalasia patients, thereby suggesting an association between achalasia and HLA-DQ1 allele, whereas TNFa11 is a marker for a protective allele of the disease, present on the B7-DRB1*1501 ancestral haplotype [26]. All these findings support a race-specific immunogenetic mechanism in the pathogenesis of idiopathic achalasia.

From another viewpoint, neuronal antibodies against Auerbach's plexus in achalasia provide an attractive hypothesis to explain this diffuse, possibly immune-based disorder [5]. In order to implicate autoimmunity in the pathophysiology of achalasia, antibodies should demonstrate the following properties: (1) disease specificity; (2) regional target specificity; (3) cellular target specificity [2]. Circulating antineuronal antibodies have been described in a variety of clinical syndromes, in addition to idiopathic achalasia (e.g., GBS [15], as well as Chagas' [27] and Parkinson's [7] diseases), including those that are associated with primary motility disorders of the gut [8]. Moreover, the concomitant appearance of GBS, Parkinson's disease and achalasia supports the notion of a viral and/or bacterial aetiology for achalasia [6,7] and suggests that apoptosis might be involved in the pathogenesis of these diseases. Importantly, as myenteric neurones synthesising nitric oxide (NO) are responsible for the inhibitory component of oesophageal peristalsis and LOS relaxation, it is considered likely that these neurones are implicated in this disease. Of note, NO triggers a programmed cell death with markers of apoptosis and is thought to participate in neuronal cell death during stroke and neurodegenerative diseases such as Parkinson's and Alzheimer's diseases, or amyotrophic lateral sclerosis. In this regard, changes of neuronal nitric oxide synthase (nNOS) expression have been linked to several human gastrointestinal disorders such as achalasia, diabetic gastroparesis and hypertrophic pyloric stenosis. They could be caused by differential transcriptional control or alternative splicing generating different nNOS proteins [28]. Essential to note that the histopathological hallmark of achalasia is degeneration of the neural elements that innervate the muscle in the oesophageal wall [2]. This event is linked with an inflammatory infiltrate (predominantly TLs) of the myenteric plexus, thereby providing evidence of an immune mediated destruction of the myenteric plexus [2], possibly through apoptotic attack of TLs to the neural elements of the oesophageal body.

H. pylori infection: apoptosis and autoimmunity

Homeostasis in the gastrointestinal mucosa is maintained by a balance between the proliferation and apoptosis of mucosal cells. Apoptosis is also implicated in various infectious and autoimmune diseases, and in carcinogenesis. *H. pylori* infection is associated with various upper gastrointestinal disorders, including chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma. It has also been implicated in a variety of extradigestive vascular conditions including ischaemic heart disease, ischaemic cerebrovascular disorders and functional vascular disorders caused by vascular dysregulation (e.g., Raynaud's phenomenon, migraine and possibly glaucoma [29,30]) and with some autoimmune conditions such as Sjögren syndrome and GBS [18], where apoptosis plays a pathogenetic role. In particular, *H. pylori* infection is associated with significant epithelial cell damage, including an increased level of apoptosis. It also induces apoptosis of fibroblasts and smooth muscle cells in lamina propria, with a concomitant reduction in the numbers of collagen fibres. These alterations may be affected by exaggerated acid secretion, decreased mucus protecting factors, and result in ulcer formation [31]. Of note, *H. pylori* is found to change the expression of genes encoding growth factors and cytokine/chemokines and their receptors, apoptosis proteins, transcriptional factors and metalloprotease-disintegrin proteins, and tissue inhibitors of metalloproteinases that contribute to the pathogen-induced gastrointestinal and extradigestive disorders [31].

Infection by *H. pylori* leads to injury of the gastric epithelium and to a cellular infiltrate of TLs by inducing activation of apoptotic surface markers on human lymphocytes and gastric epithelial cells and/or gastric adenocarcinoma cell lines [31]. Moreover, *H. pylori* appears to upregulate the expression of HLA class II (HLA-DR) molecules on gastric epithelial cells and induces their apoptosis, probably through activation of TLs [31]. 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 [31]. Virulent factors possessed by *H. pylori* that promote colonisation (urease) bind to class II major histocompatibility complex on gastric epithelial cells and induce their apoptosis [31]. 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 [13,32]. From another viewpoint, gastric epithelial cells are capable to express Fas and its ligand (FasL), thereby controlling the inflammatory process by inducing apoptotic cell death to Fas-bearing infiltrating lymphocytes [13,32]. Taken together, these data support the interplay between *H. pylori* and epithelial cells in the course of *H. pylori*-mediated apoptotic gastropathy.

Besides, *H. pylori* infection induces gastrin levels to increase that in turn increase the levels of NO. NO is a rapidly diffusing gas and in sufficient concentrations is a potent neurotoxin that may facilitate the apoptotic death of ganglion cells in neuropathies [31], possibly including achalasia. Similarly, other *H. pylori* induced cytokines, such as TNF-α may exert apoptotic and/or antiapoptotic effects at distance, thereby playing a potential role in the pathogenesis of extraintestinal vascular disorders including migraine, Raynaud's phenomenon, ischaemic heart disease, glaucoma disease [29–31] and probably other neuropathies including achalasia.

The association between *H. pylori* infection and gastric autoimmunity is now well established [10]. Patients infected with *H. pylori* have been shown to possess autoantibodies that cross-react with antigens expressed on the gastric mucosa [33]. The gastric [H(+)/K(+)] ATPase located in canaliculi of parietal cells appears to be a target of this autoimmune response. The presence of autoantibodies, in particular those directed to parietal cells, correlates with histological and clinical parameters of gastric mucosa atrophy [34]. Therefore, *H. pylori*-autoimmunity may play a critical role in the pathogenesis of chronic atrophic gastritis, a known risk factor for gastric cancer. It has been suggested, that molecular mimicry between *H. pylori* and the host on the level of Lewis x and Lewis y blood group antigens leads to the development of these autoantibodies [35]. *H. 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 [31,36].

Interestingly, molecular mimicry of host structures by the saccharide portion of lipopolysaccharides of the gastrointestinal pathogens *Campylobacter jejuni* (*C. jejuni*) and *H. pylori* is

thought to be connected with the development of autoimmune sequelae observed in neuropathies. *C. jejuni*, a principal cause of gastroenteritis, is the most common antecedent infection in GBS, an inflammatory autoimmune neuropathy. Chemical analyses of the core oligosaccharides of neuropathy-associated *C. jejuni* strains have revealed structural homology with human gangliosides. Serum antibodies against gangliosides are found in one third of patients with GBS, but are generally absent in enteritis cases. Collective data suggest that the antibodies are induced by antecedent infection with *C. jejuni*, and subsequently react with nerve tissue causing damage [37], possibly by apoptosis. In addition, several IgG antibodies against *H. pylori* proteins are found in the cerebrospinal fluid in 57% of patients with GBS. No cross reactivity against *C. jejuni* is observed and these antibodies may also be involved in the immune responses of patients with GBS [15]. Similarly, 46% of patients with GBS have specific IgG antibodies to VacA of *H. pylori* in the cerebrospinal fluid, and the sequence homology found between VacA and human [Na(+)/K(+)] ATPase A subunit suggests that antibodies to VacA involve ion channels in abaxonal Schwann cell plasmalemma resulting in demyelination in some patients within the cerebrospinal fluid [17]. In this regard, it is relevant to speculate that such anti-*H. pylori*-mediated apoptotic mechanisms might also lead to degeneration of ganglion cells in the oesophageal body, thereby inducing achalasia.

Concluding comments

Considering all above-mentioned data, we can speculate that various apoptotic signals induced by *H. pylori* appear to influence the ENS, thereby indicating an underlying dysregulation of apoptosis as a pathophysiological link between *H. pylori* infection and idiopathic achalasia. Since achalasia is a long recognised clinical entity affecting mainly older ages with a poorly understood underlying pathophysiology and *H. pylori* infection prevalence also increases with age having a worldwide distribution, further research is necessary to elucidate how exactly abnormal regulation of *H. pylori*-mediated apoptosis may influence the pathogenesis of idiopathic achalasia. Moreover, there are many similarities found between gastric autoimmunity caused by *H. pylori* and achalasia. It would be interesting to hypothesise that *H. pylori*-induced apoptosis by autoantibodies through molecular mimicry may not be limited only in myenteric neurones of the oesophageal body, but appears to be a more general process involving several tissues that include specific neurones. Whether eradication of *H. pylori* infection may indirectly offer benefit to the pathophysiology of idiopathic achalasia by ameliorating the apoptotic loss of ganglion cells and their axons in the oesophageal wall remains to be elucidated.

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Induction of apoptosis as a proposed pathophysiological link between glaucoma and *Helicobacter pylori* infection

Jannis Kountouras*, Christos Zavos, Dimitrios Chatzopoulos

Department of Gastroenterology, 2nd Medical Clinic, Ippokration Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece

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Summary Although apoptosis is equally important both for the development and for the maintenance of homeostasis in some adult tissues, it can also be associated with disease processes. Current studies indicate that apoptosis is a mechanism of cell death in several important ocular and gastrointestinal diseases including glaucoma, and *Helicobacter pylori* (*H. pylori*)-induced upper gastrointestinal disorders and/or extraintestinal diseases, including autoimmune and neurodegenerative ones (Sjögren's syndrome, Guillain-Barre syndrome or Parkinson's disease). Glaucoma is also associated with similar autoimmune and neurodegenerative disorders, characterized by apoptotic loss of specific populations of neurons. Recently, a high prevalence of *H. pylori* infection has been recognized in patients with chronic open-angle glaucoma. In addition, *H. pylori* eradication may positively influence glaucoma parameters, thereby suggesting a possible causal link between *H. pylori* and glaucoma. *H. pylori* infection may influence the pathophysiology of glaucoma by releasing various proinflammatory and vasoactive substances, as well as by influencing apoptotic process; parameters that may also exert their own effects in the induction and/or progression of glaucomatous neuropathy. Importantly, *H. pylori* infection and glaucoma share the Fas/FasL and the mitochondria-mediated apoptotic pathways, thereby proposing an apoptotic link in the pathophysiology of both diseases.

Apoptosis is considered a physiological form of cell death that involves the active participation of the dying cell in its demise [1–3]. It has been described as a pathway of developmental cell elimination in different tissues, including the retina and the gastrointestinal epithelium, allowing for the safe disposal of cellular remnants without causing inflammatory harm to the surrounding tissue [4].

Apoptotic stimuli vary and include internal and external activators, trophic factor deprivation and cellular damage. Physical stimuli can also induce apoptosis. Irradiation with ultraviolet light, for example, mediates apoptotic nuclear strand cleavage [5].

In general, there are two ways in which cells die. They are either killed by harmful toxic and mechanical agents (necrosis) or they are induced to commit suicide [6,7]. Necrosis is a rapid process that involves cell swelling, plasma membrane fracture, nuclear and mitochondrial disruption, rapid ATP depletion, cytoplasmic extrusion and

*Corresponding author. Present address: 8 Fanariou St., Byzantio 551 33, Thessaloniki, Macedonia, Greece. Tel.: +30-2310-892238; fax: +30-2310-992794.
E-mail address: jannis@med.auth.gr (J. Kountouras).

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Abbreviations	Explanation
IL	interleukin
FLICE	Fas-associated death-domain-containing protein-like interleukin-1β-converting enzyme
ICE	caspases or interleukin-1α-converting enzyme
PARP	poly ADP-ribose polymerase
H. pylori	*Helicobacter pylori*
TNF	tumor necrosis factor
NO	nitric oxide
iNOS	inhibitor of nitric oxide synthase
Hsps	heat shock proteins

inflammation. In contrast, apoptosis (a form of genetically programmed cell death that is often referred to as a form of cellular suicide) is a slower subacute process that involves cell shrinkage, membrane blebbing, nuclear chromatin condensation, nuclear DNA cleavage by endonucleases, maintenance of organelles and plasma membrane integrity, loss of plasma membrane phospholipid symmetry, membrane-wrapped cytoplasmic and nuclear bodies, and cysteine protease (caspase) activation [8,9]. Notably, an essential aspect of apoptosis entails parallel activation of cellular proteases and endonucleases. Early recruitment of an "initiator" protease [e.g., granzyme B, Fas-associated death-domain-containing protein-like interleukin (IL)-1β-converting enzyme (FLICE)] leads to proteolytic activation of "amplifier" proteases [e.g., caspases or IL-1β-converting enzyme (ICE)], which, in turn, activate several families of so-called "machinery" proteases (e.g., CPP32/ICE-LAP6/caspase-3); the latter group of enzymes finally mediates proteolytic degradation of the cytoskeleton and other substrates [e.g., lamins, poly ADP-ribose polymerase (PARP)] in the final stages of apoptosis. During apoptotic proteolysis, genomic DNA is cleaved into progressively smaller pieces (e.g., ~300-kb rosettes, ~50 kb loops, and finally a "laddered" spectrum of oligonucleosomal fragments in integer-multiples of ~180–200 bp), thereby removing the cells with damaged DNA [3].

Although it is equally important both for the development and for the maintenance of homeostasis in some adult tissues, apoptosis can also be associated with disease processes [8,10,11]. Current studies indicate that apoptosis is a mechanism of cell death in several important ocular and gastrointestinal diseases, including glaucoma, retinitis pigmentosa, cataract formation, retinoblastoma, retinal ischemia, diabetic retinopathy and *Helicobacter pylori* (*H. pylori*)-induced upper gastrointestinal disorders and/or extraintestinal diseases, including autoimmune and neurodegenerative ones (Sjögren's syndrome, Guillain-Barre syndrome or Parkinson's disease) [12,13]. Glaucoma is also associated with similar autoimmune and neurodegenerative disorders, such as Guillain-Barre syndrome, Alzheimer's disease or Parkinson's disease, characterized by apoptotic loss of specific populations of neurons [14,15].

Recently, we have documented a high prevalence of *H. pylori* infection in patients with chronic open-angle glaucoma [16]. In addition, *H. pylori* eradication may positively influence glaucoma parameters, thereby suggesting a possible causal link between *H. pylori* and glaucoma [17]. *H. pylori* infection may influence the pathophysiology of glaucoma by promoting platelet and platelet-leukocyte aggregation, releasing proinflammatory and vasoactive substances, such as cytokines [IL-1, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor (TNF)-α, interferon-γ], eicosanoids (leukotrienes, prostaglandins) and acute phase proteins (fibrinogen, C-reactive protein) [18], involved in a number of vascular disorders including glaucoma, stimulating mononuclear cells to induce a tissue factor-like procoagulant activity that converts fibrinogen into fibrin, causing the development of cross mimicry between endothelial and *H. pylori* antigens, producing reactive oxygen metabolites and circulating lipid peroxides, and influencing the apoptotic process that may also be involved in the pathogenesis of glaucomatous neuropathy. In particular, increased endothelin-1 (a potent constrictor of arterioles and venules), nitric oxide (NO) and inhibitor of nitric oxide synthase (iNOS) levels are associated with *H. pylori* infection [19,20]. Endothelin-1-induced vasoconstriction of the anterior optic nerve vessels and NO modulation of vascular tone in the ophthalmic artery may produce glaucomatous damage [21]. Moreover, NO is a rapidly diffusing gas and a potent neurotoxin that may facilitate the apoptotic death of retinal ganglion cells in glaucomatous optic neuropathy [22]. Support for the consideration of NO neurotoxicity in glaucoma is provided by experimental evidence demonstrating that retinal ganglion cell apoptosis is attenuated by neutralizing antibodies against TNF-α or by selective inhibitors of inducible NOS, thereby suggesting that the inhibitions of TNF-α or the inducible isoform NOS2 may provide novel therapeutic targets for neuroprotection in the treatment of glaucomatous optic neuropathy [23]. Similarly, *H. pylori*-induced cytokines, such as TNF-α may exert apoptotic and/or antiapoptotic effects at distance, thereby playing a potential role in the

pathogenesis of extraintestinal vascular disorders including migraine, Raynaud's phenomenon, ischemic heart disease and possibly glaucoma disease [24,25].

Importantly, autoimmune mechanisms may be responsible for eliciting glaucomatous damage to the optic nerve [26], and the evidence that autoantibodies to proteins in retina and/or optic nerve may contribute to glaucomatous optic neuropathy is consistent with a wider literature in medicine, implicating a causative role for autoantibodies in many peripheral neuropathies including Guillain-Barre syndrome. The autoantibodies directed toward retinal antigens may be involved in facilitating apoptotic cell death in glaucoma patients [27]. Similarly, gastric autoimmunity is also well established in patients with *H. pylori* infection associated with induction of autoantibodies that cross-react with the gastric mucosa [28]. This type of autoreactivity is linked with the presence and degree of inflammation and atrophy of the glands [29]. Moreover, molecular mimicry of host structures by the saccharide portion of lipopolysaccharides of *H. pylori* is thought to be connected with the development of autoimmune sequelae (autoantibodies) in neuropathies (such as Guillain-Barre syndrome [30] or possibly glaucoma), that induce apoptotic damage of neurons [31]. Support for this theory is provided by our recent observations indicating that the titer of anti- *H. pylori* IgG antibodies in the aqueous humor of patients with glaucoma may reflect the severity of glaucomatous damage [32]. The most likely mechanism for the role of this organism is via molecular mimicry autoimmune sequelae. An attractive, although rather gross, correlation is the observation that the positivity status for *H. pylori* appeared to correlate with the severity of glaucomatous cupping in our glaucoma patients [32]. Further studies, however, are needed to support the hypothesis that the presence of IgG antibodies to *H. pylori* may adversely influence progression of glaucomatous neuropathy.

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 host's immune gastric tissue [33,34]. 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. 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 Sjögren's syndrome, and other vascular disorders, including atherosclerosis, coronary heart disease, and possibly glaucoma, or even more MALT lymphoma must be considered. In the case of glaucoma, there is convincing evidence that the presence of increased serum autoantibodies against hsp-27 may have pathogenetic importance [23]. Indeed, exogenously applied hsp-27 antibody enters, through parapapillary defects (i.e., atrophy of retinal pigment epithelium close to the optic nerve head) of the outer blood–retina barrier, to neuronal cells in human retina, by an endocytic mechanism. After internalization, hsp-27 antibody facilitates apoptotic cell death as characterized by morphological assessment, DNA fragmentation, and the activation of caspases. In particular, hsp-27 antibody binding to actin results in depolymerization and proteolytic cleavage of actin in a dose-dependent manner, thereby inactivating or attenuating the ability of native hsp-27 to stabilize actin cytoskeleton, and impairing cell survival in glaucoma [23]. The observations that the protective activity of native hsp-27 can be modulated by autoantibodies to hsp-27, and the apoptotic cell death of retinal ganglion cells can be induced by intravitreal administration of purified anti-neuron specific enolase antibody, may provide a rationale for novel immune-based strategies to modulate apoptotic cell death in glaucoma [23].

In view of these data, we can speculate that variable apoptotic signals induced by *H. pylori* appear to influence the glaucomatous optic neuropathy, thereby indicating an underlying dysregulation of apoptosis as a pathophysiological link between *H. pylori* infection and glaucoma. Since glaucoma is currently the second commonest cause of blindness in the world, and *H. pylori* is also a common infection with worldwide distribution, further research is necessary to elucidate how exactly abnormal regulation of *H. pylori*-mediated apoptosis may influence the pathogenesis of glaucoma. Moreover, if eradication of *H. pylori* infection may indirectly offer benefit to the glaucomatous optic neuropathy (which accounts for approximately 90.8 million affected individuals) by ameliorating the apoptotic loss of retinal ganglion cells and their axons, and the progressive loss of visual field sensitivity, remains to be elucidated.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• *H pylori* •

Effects of *Helicobacter pylori* infection on gastric emptying rate in patients with non-ulcer dyspepsia

Grigoris I Leontiadis, George I Mimopoulos, Efstratios Maltezos, Stamatia Kotsiou, Konstantinos I Manolas, Konstantinos Simopoulos, Dimitrios Hatseras

Grigoris I Leontiadis, Efstratios Maltezos, 2nd Department of Internal Medicine, Democritus University of Thrace, Alexandroupolis, Greece
George I Mimopoulos, Department of Endoscopic Surgery, Democritus University of Thrace, Alexandroupolis, Greece
Stamatia Kotsiou, 1st Department of Internal Medicine, Democritus University of Thrace, Alexandroupolis, Greece
Konstantinos I Manolas, 1st Department Surgery, Democritus University of Thrace, Alexandroupolis, Greece
Konstantinos Simopoulos, 2nd Department of Surgery, Democritus University of Thrace, Alexandroupolis, Greece
Dimitrios Hatseras, Department of Cardiology, Democritus University of Thrace, Alexandroupolis, Greece
Correspondence to: Dr. Grigoris I Leontiadis, 4 Karaiia Street, Thessaloniki 546 44, Greece. grleo@yahoo.com
Telephone: +30-2310-323535 **Fax:** +30-2510-323535
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Abstract

AIM: The pathogenesis of delayed gastric emptying in patients with non-ulcer dyspepsia (NUD) remains unclear. We aimed to examine whether gastric emptying rate in NUD patients was associated with *Helicobacter pylori* (*H pylori*) infection and whether it was affected by eradication of the infection.

METHODS: Gastric emptying rate of a mixed solid-liquid meal was assessed by the paracetamol absorption method in NUD patients and asymptomatic controls (n=17). *H pylori* status was assessed by serology and biopsy urease test. *H pylori*-positive NUD patients (n=23) received 10-day triple eradication therapy. *H pylori* status was re-assessed by biopsy urease test four weeks later, and if eradication was confirmed, gastric emptying rate was re-evaluated.

RESULTS: Thirty-three NUD patients and 17 controls were evaluated. NUD patients had significantly delayed gastric emptying compared with controls. The mean maximum plasma paracetamol concentration divided by body mass (Cmax/BM) was 0.173 and 0.224 mg/L·kg respectively (P=0.02), the mean area under plasma paracetamol concentration-time curve divided by body mass (AUC/BM) was 18.42 and 24.39 mg·min/L·kg respectively (P=0.01). Gastric emptying rate did not differ significantly between *H pylori*-positive and *H pylori*-negative NUD patients. The mean Cmax/BM was 0.172 and 0.177 mg/L·kg respectively (P=0.58), the mean AUC/BM was 18.43 and 18.38 mg·min/L·kg respectively (P=0.91). Among 14 NUD patients who were initially *H pylori*-positive, confirmed eradication of the infection did not significantly alter gastric emptying rate. The mean Cmax/BM was 0.171 and 0.160 mg/L·kg before and after *Hp* eradication, respectively (P=0.64), the mean AUC/BM was 17.41 and 18.02 mg·min/L·kg before and after eradication, respectively (P=0.93).

CONCLUSION: Although gastric emptying is delayed in NUD patients compared with controls, gastric emptying rate is not associated with *H pylori* status nor it is affected by eradication of the infection.

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INTRODUCTION

Dyspepsia is defined as pain or discomfort centered in the upper abdomen, according to the Rome II criteria[1]. Discomfort refers to a subjective, unpleasant feeling that the patient does not interpret as pain and which can include any of the following: upper abdominal fullness, early satiety, bloating, or nausea. Functional or non-ulcer dyspepsia (NUD) is defined as persistent or recurrent dyspepsia for at least 12 wk, which need not to be consecutive, within the preceding 12 mo of persistent or recurrent dyspepsia, with no evidence of organic disease that is likely to explain the symptoms, and no evidence that symptoms are exclusively relieved by defecation or associated with the onset of a change in stool frequency or stool form (*i.e.*, not irritable bowel syndrome)[1].

NUD is a common healthcare problem: The estimated annual prevalence in Western countries is 15%[2]. Despite a great deal of scientific attention, little is known about the pathogenesis of NUD. *Helicobacter pylori* (*H pylori*) infection and delayed gastric emptying have both been documented to have higher prevalence in patients with NUD compared with asymptomatic controls[3-6]. However, it is unknown if *H pylori* infection and/or delayed gastric emptying are involved in the pathogenesis of NUD. Furthermore, it is still unclear whether there is a causal association between these two factors in patients with NUD.

Therefore, we designed this study to investigate whether gastric emptying rate in patients with NUD was associated with the presence of *H pylori* infection, and whether the eradication of the infection affected the gastric emptying rate in these patients. We also sought to confirm in our population the well-known finding of delayed gastric emptying in patients with NUD compared with asymptomatic controls.

MATERIALS AND METHODS

Subjects

Consecutive patients with dyspeptic symptoms who attended the self-referred out-patient clinic of the 2nd Department of Internal Medicine, General Regional Hospital of Alexandroupolis were candidates for inclusion in the study. Inclusion criteria were: (1) age between 18 and 65 years; (2) presence of dyspeptic symptoms, defined as upper abdominal pain, upper abdominal discomfort, upper abdominal fullness, early satiety, or nausea/vomiting, continuously or intermittently for at least three mo; (3) no relevant findings revealed by physical examination other

than epigastric tenderness; (4) normal gastroscopy except mild erythema of gastric or duodenal mucosa; (5) normal abdominal ultrasound scan; (6) normal full blood count, liver function tests, fasting glucose, urea, creatinine, electrolytes, amylase and thyroid function tests. Exclusion criteria were: (1) presence of any organic or psychiatric disease that could affect the evaluation, treatment or compliance of the patient; (2) heartburn as the predominant symptom; (3) predominant symptoms being compatible with the diagnosis of irritable bowel syndrome or functional constipation as defined by Rome I criteria[35]; (4) pregnancy or breast feeding; (5) chronic alcoholism or drug abuse; (6) past medical history of peptic ulcer disease, oesophagitis, pancreatitis, abdominal trauma, or abdominal surgery other than uncomplicated appendectomy; (7) intake of antibiotics 4 wk prior to inclusion; (8) regular intake of non steroidal anti-inflammatory drugs, including aspirin, or other medications that could cause dyspepsia during the 3 mo prior to inclusion.

The asymptomatic controls were recruited from the employees of General Regional Hospital of Alexandroupolis. Eligibility criteria were: (1) age between 18 and 65 years; (2) absence of dyspeptic symptoms; (3) absence of any organic or psychiatric disease that could affect the evaluation, treatment or compliance of the participant; (4) intake of antibiotics 4 wk prior to inclusion.

Proton pump inhibitors and H_2-receptor antagonists were withheld from all participants for 7 d before gastroscopy or gastric emptying testing. Paracetamol and all medications with any known potential effect on gastrointestinal motility were withheld for, respectively, two and three days before gastric emptying testing.

Study protocol

Study procedures were timed as follows: NUD patients underwent gastroscopy and two biopsy specimens were collected, one from the antrum and one from the corpus, for urease testing for *H pylori* (*CLOtest®, Delta West Ltd, Australia*). The presence of *H pylori* IgG antibodies in serum was assessed by ELISA (*H pylori GVE 57311 IgG, PLK, Italy*). On the following day, gastric emptying rate was assessed by the paracetamol absorption method as described below. *H pylori* -positive NUD patients received 10 d of *H pylori* eradication therapy consisting of omeprazole 20 mg twice daily, amoxicillin 1 000 mg twice daily and clarithromycin 500 mg twice daily. Four weeks later, *H pylori* status was re-assessed by biopsy urease test. If eradication of *H pylori* infection was confirmed, gastric emptying rate was re-evaluated. Asymptomatic controls were tested for *H pylori* infection by serology (*H pylori* IgG ELISA) and had gastric emptying rate assessed by the paracetamol absorption method.

The study protocol conformed to the World Medical Association Helsinki Declaration in 1964 as amended in 1996 and was approved by the regional Ethics Committee. Informed consent was obtained from all study subjects. This work was not financially supported by any outside agency or pharmaceutical company.

Assessment of gastric emptying rate

Gastric emptying rate was assessed by the paracetamol absorption method, which was conceived by Heading *et al*[36,37] and recently re-evaluated[38,39]. The test meal was administered following overnight fasting and consisted of: (1) three cream crackers (total mass is 35 g); (2) a 200-mL carton of *Fortimel®* (*Nutricia Clinical, Holland*; liquid nutritional supplement; 410 mOsm/L; 4.184×10^3 kJ/L; containing 19.4 g of protein, 4.2 g of fat and 20.8 g of carbohydrates per carton); (3) 1 g of paracetamol as two tablets 500 mg *Depon®, Bristol-Myers Squibb*; (4) 100 mL of tap water. Peripheral venous blood samples were obtained *via* a heparinised cannula immediately after the ingestion of paracetamol (time 0) and at 30 min intervals over a 3-h period (7 samples in total). During the 3-h test period, the subjects were free to sit, stand or walk; they were not allowed to eat, drink or smoke.

Paracetamol plasma concentration was determined with the *TDxFLx® System (Abbott, IL, USA)* by using fluorescence polarization immunoassay technology. The following parameters were calculated: time to detect paracetamol in plasma (lag phase), maximum plasma paracetamol concentration divided by body mass (C_{max} /BM); time to reach maximum plasma paracetamol concentration (T_{max}), area under the plasma paracetamol concentration – time curve divided by body weight (AUC/BM).

Primary outcomes were the absolute difference in gastric emptying rate as assessed by Cmax/BW and AUC/BW in *H pylori*-positive and *H pylori*-negative NUD patients, and in *H pylori*- positive NUD patients before and after eradication.

Secondary outcomes were the absolute difference in gastric emptying rate as assessed by Cmax/BM and AUC/BM in NUD patients compared to asymptomatic controls. We also assessed gastric emptying lag phase and T_{max} in all study groups.

Sample size calculation

Sample size was calculated *a priori* with the Altman nomogram[39]. A pilot study of five *H pylori*-positive NUD patients provided an estimate of mean C_{max}/BM (19.0 mg· min/L· kg) and standard deviation (5.0 mg· min/L· kg). We calculated that at least 10 subjects were required in each study group (*H pylori*-negative NUD patients, *H pylori*-positive NUD patients, post-eradication NUD patients, asymptomatic controls) in order to detect a clinically important relative difference (*a priori* set as ≥ 25%) between two study groups or within person comparison before and after *H pylori* eradication ($1-\beta=0.8$, $\alpha=0.05$, two tailed significance).

Statistic analysis

Statistical analysis was carried out according to a pre-established analysis plan. Proportions were compared by χ^2 test with Fisher's exact test when appropriate. Regarding continuous outcomes, study groups were compared by the Mann-Whitney *U* test, while differences before and after *H pylori* eradication were assessed by Wilcoxon matched pairs signed rank test. Two sided significance tests were used throughout. The software used was Statistical Package for the Social Sciences 10.0.1 for Windows (release 1999, SPSS Inc., Chicago, IL). Confidence intervals were calculated with Confidence Interval Analysis software 2.0.0 (release 2000, T. Bryant, Univ. of Southampton).

RESULTS

Thirty-three patients with NUD and 17 asymptomatic volunteers were included in the study during two recruitment periods (January to March 1997 and March to April 1998). All participants were Caucasian. Twenty-three of the NUD patients (70%) had both a positive serum anti-*H pylori* IgG and biopsy urease test and were considered *H pylori*- positive. The remaining 10 NUD patients had both tests negative and were considered *H pylori*- negative. The baseline characteristics of the study groups are displayed in Table 1. The only significant difference found was a difference in age between control group and NUD group ($P=0.02$). Nevertheless multiple regression (ANOVA) revealed that age had no effect on any of the study outcomes. There was, therefore, no need for adjustment for age.

All study subjects underwent evaluation of gastric emptying. There was no significant difference in gastric emptying rate between *H pylori*-positive and *H pylori*-negative NUD patients as assessed by the primary study parameters C_{max}/BM and

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AUC/BM (Table 2). NUD patients had significantly delayed gastric emptying compared with asymptomatic controls as assessed by C_{max} /BM and AUC/BM (Table 3). Gastric emptying lag phase and T_{max} did not differ significantly in any comparison (data not shown).

All 23 *H pylori*-positive NUD patients received eradication therapy. Of those, 18 completed the 10-d course and 4 wk later underwent repeat gastroscopy and biopsy urease test, eradication was confirmed in 14 patients (per protocol eradication rate 77.8%). Of the remaining five patients who received eradication therapy, 4 did not consent to repeat gastroscopy and 1 discontinued the course on the third day due to severe diarrhea (*C. difficile* toxin negative). No other side effects of the eradication regimen were reported on direct questioning.

All 14 NUD patients with confirmed *H pylori* eradication underwent repeat assessment of gastric emptying. No significant difference was found in the gastric emptying parameters C_{max} /BM and AUC/BM compared with the values prior to eradication (intra-individual comparisons) as shown in Table 4. Neither gastric emptying lag phase nor T_{max} differed significantly compared with the values prior to eradication (data not shown).

DISCUSSION

This study confirmed that gastric emptying is significantly delayed in patients with NUD compared to asymptomatic controls (Table 3). This has been well established for years[3,11]. In fact, our results showed that the 95% confidence interval (CI) for the absolute difference between the medians of gastric emptying parameters, C_{max} /BW and AUC/BW, was compatible with a relative difference encompassing the predefined limit of clinical importance (*i.e.* 25%). Consequently, our results need to be interpreted as evidence of a statistically significant difference in gastric emptying rate between patients with NUD and asymptomatic controls, which however is unclear if it is clinically relevant. The above comparison was not the primary outcome of our; Nonethelss the fact that these results are in agreement with the current medical literature corroborates the methodological validity of our study and the credibility of the main outcomes.

The pathoptysiology of delayed gastric emptying in patients with NUD, as well as its clinical implications, remains unknown. *H pylori* gastritis - which was also more prevalent in patients with NUD[11] - has been suggested as a causal factor for the gastric motility disorders found in these patients. There is no solid evidence supporting this hypothesis although there is some biological plausibility since a mucosal inflammatory reaction could affect the function of enteric nerves and smooth muscle[12].

In our attempt to investigate the potential role of *H pylori* in the above mentioned motility disorders, we found that the gastric emptying rate in patients with NUD did not differ significantly between *H pylori*-positive and *H pylori*-negative patients (Table 2). Furthermore, eradication of *H pylori* infection in patients with NUD did not induce any significant modification

Table 1 Baseline characteristics of patients with non-ulcer dyspepsia (NUD)

Characteristics	Controls (n=17)	All NUD pts (n=33)	Hp positive NUD pts (n=23)	Hp negative NUD pts (n=10)
Sex (Male:Female)	9:8	20:13	13:10	7:3
Age (yr) (mean±SD)	27.7±6.7	37.3±14.5	39.3±14.2	35.2±18.4
Body mass (kg) (mean±SD)	66.6±13.8	74.3±10.0	75.6±9.8	71.2±10.1
Hp(+) (%)	9 (52.9)	23 (69.7)	23 (100)	0 (0)
Smokers (%)	8 (47.1)	10 (30.3)	7 (30.4)	3 (30)

Table 2 Gastric emptying: *H pylori*-positive NUD patients *vs H pylori*-negative NUD patients

parameters (mean±SD, M)	*Hp* positiveNUD pts (n=23)	*Hp* negative NUD pts (n=10)	Mann-Whitney U test	95% CI for difference between medians (*Hp* pos. – *Hp* neg.)
Cmax/BM(mg/L·kg)	0.172±0.070 (0.153)	0.177±0.050 (0.176)	P=0.58	-0.050 to 0.037
AUC/BM(mg·min/L·kg)	18.43±6.95 (18.53)	18.38±5.94 (19.42)	P=0.91	-5.36 to 4.62

C_{max} /BM: maximum plasma paracetamol concentration divided by body mass; AUC/BW: area under the plasma paracetamol concentration-time curve divided by body mass. M: median.

Table 3 Gastric emptying: NUD patients *vs* asymptomatic controls

Parameters (mean±SD, M)	NUD pts (n=33)	Controls (n=17)	Mann-Whitney U test	95% CI for difference between medians (NUD pts-Controls)
Cmax/BM (mg/L·kg)	0.175±0.064 (0.163)	0.224±0.076 (0.221)	P=0.02	-0.012 to-0.095
AUC/BM (mg·min/L·kg)	18.42±6.56 (18.53)	24.34±8.06 (25.53)	P=0.01	-2.11 to-10.93

C_{max} /BM: maximum plasma paracetamol concentration divided by body mass; AUC/BM: area under the plasma paracetamol concentration – time curve divided by body mass. M: median.

Table 4 Gastric emptying: *H pylori*-positive NUD patients before and after eradication (intra-individual comparisons)

Parameters (mean±SD, M)	Initially *Hp* positive NUD pts (n=14)		Wilcoxon matched pairs test	95%CI for the median difference (after – before)
	Before eradication	After eradication		
Cmax/BM (mg/L·kg)	0.171±0.074 (0.167)	0.160±0.064 (0.146)	P=0.64	-0.047 to 0.278
AUC/BM (mg·min/L·kg)	17.41±5.25 (18.95)	18.02±7.25 (16.88)	P=0.93	-3.04 to 4.38

C_{max} /BM: maximum plasma paracetamol concentration divided by body mass. AUC/BM: area under the plasma paracetamol concentration–time curve divided by body mass. M: median.

of the gastric emptying rate (Table 4). However, for both comparisons, the 95% CI for the absolute difference between the medians of gastric emptying parameters was marginally compatible with the pre-defined level of a clinically important relative difference of 25%. This should be interpreted, therefore, as insufficient evidence to confirm or exclude a clinically important difference in gastric emptying rate between *H pylori*-positive and *H pylori*-negative patients with NUD. The same conclusion applies to the changes of gastric emptying rate following eradication of *H pylori* infection in patients with NUD.

Our results are in agreement with most previous publications, which did not detect any influence of *H pylori* status on gastric emptying rate in patients with NUD[13-15]. However, it is possible that some of these studies were not adequately powered to detect the targeted difference. Some investigators were able to demonstrate significant differences, but their conclusions differed. For example, Fock *et al*.[14] found that gastric emptying was slower in *H pylori*-positive NUD patients compared to *H pylori*-negative NUD patients, while Tucci *et al*.[13] found the opposite. No meta-analysis has yet addressed this issue.

Regarding the effect of the eradication of *H pylori* infection on gastric emptying rate in NUD patients, our results are in line with three other trials, which were unable to detect any changes in gastric emptying rate after a follow up period of one[26], six[17] and 12 mo[25]. Nonetheless, other investigators found that eradication of the infection significantly increased gastric emptying rate after a follow-up of 1 mo[16] or "normalised" previously abnormal (*i.e*. rapid or delayed) gastric emptying after a 2-mo follow up[18]. There was considerable methodological heterogeneity among these trials, which complicates any attempts to draw a conclusive answer.

Further larger trials would be helpful. These would be facilitated by using safe and non-invasive methods of gastric emptying assessment, such as the paracetamol absorption method, ultrasonography[11], or ^{13}C octanoic acid breath test. Utilization of urea breath testing for confirmation of *H pylori* eradication[11], which was not available to us at the time of the research, might improve the compliance of participants and could allow investigators to lengthen the post-eradication follow-up time without a significant dropout rate. Future studies should also explore whether specific *H pylori* characteristics, such as CagA phenotype[19], or differences in host response are implicated in the pathogenesis of gastric emptying disorders in patients with NUD.

In conclusion, gastric emptying is delayed in patients with NUD but is unrelated to *H pylori* status. Eradication of *H pylori* infection in *H pylori*-positive patients with NUD does not significantly alter the gastric emptying rate. Although our results are not definitive, they will contribute to a better understanding of the pathogenesis of NUD - especially when quantitatively synthesised with analogous data in future meta-analyses.

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Effect of *Helicobacter pylori* Infection on the Risk of Upper Gastrointestinal Bleeding in Users of Nonsteroidal Anti-inflammatory Drugs

George V. Papatheodoridis, MD, Despina Papadelli, MD, Evangelos Cholongitas, MD, Dimitrios Vassilopoulos, MD, Andreas Mentis, MD, Stephanos J. Hadziyannis, MD

PURPOSE: We evaluated whether infection with *Helicobacter pylori*, including specific cytotoxic-associated antigen (CagA)-positive strains, increase the risk of upper gastrointestinal bleeding in users of nonsteroidal anti-inflammatory drugs (NSAIDs).

METHODS: Cases with upper gastrointestinal bleeding and recent NSAID use, including aspirin, who were admitted during 2001, were compared with age- and sex-matched outpatient controls who had recent NSAID use. *H. pylori* infection was diagnosed by serum antibodies or the ^{13}C-urea breath test; and CagA seropositivity was diagnosed by enzyme-linked immunoassay.

RESULTS: *H. pylori* was detected significantly more frequently in cases of bleeding than controls (79% [63/80] vs. 56% [45/80], $P = 0.004$). Cases of bleeding were more likely than controls to have a history of peptic ulcer (34% [n = 27] vs. 13% [n = 10], $P = 0.003$), previous upper gastrointestinal bleeding (19% [n = 15] vs. 6% [n = 5], $P = 0.03$), recent dyspepsia (29% [n = 23] vs. 15% [n = 12], $P = 0.06$), and <3 months of NSAID use (58% [n = 46] vs. 40% [n = 32], $P = 0.04$). CagA positivity was not associated with gastrointestinal bleeding. In a multivariate analysis, *H. pylori* infection was the only significant risk factor for upper gastrointestinal bleeding (odds ratio = 1.7; 95% confidence interval: 1.2 to 2.5; $P = 0.004$).

CONCLUSION: *H. pylori* infection almost doubles the risk of upper gastrointestinal bleeding among users of NSAIDs. **Am J Med. 2004;116:601–605.** ©2004 by Excerpta Medica Inc.

Gastrointestinal complications, particularly peptic ulcer and erosions, are the most common adverse events among users of nonsteroidal anti-inflammatory drugs (NSAIDs) (1–3), whereas *Helicobacter pylori* infection is the most frequent cause of peptic ulcer disease in patients not taking NSAIDs (4). However, the effects of the simultaneous presence of both *H. pylori* infection and NSAID use on the risks of peptic ulcer and its complications are uncertain (5–13).

Although infection with *H. pylori* causes gastric inflammation in all persons who are infected with the organism (4), the host response and the clinical manifestations of infection appear to be associated with genetic differences in the bacterial strain (4,14,15). For example, the risk of development of peptic ulcer disease is higher in persons infected with cytotoxic-associated antigen (CagA)-positive strains than with CagA-negative strains (16). (CagA is a bacterial antigen, to which antibodies develop.)

From the Academic Department of Medicine (GVP, DP, EC, DV), Hippokration General Hospital of Athens; Hellenic Pasteur Institute (AM); and Department of Medicine and Hepatology (SJH), Henry Dunant Hospital, Athens, Greece.

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Requests for reprints should be addressed to George V. Papatheodoridis, MD, Academic Department of Medicine, Hippokration General Hospital of Athens, 114 Vas. Sophias Avenue, 115 27 Athens, Greece, or gpapath@cc.uoa.gr.

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We sought to assess the effect of infection with *H. pylori*, and CagA status, on the risk of upper gastrointestinal bleeding in users of NSAIDs.

METHODS

All patients with acute upper gastrointestinal bleeding (cases of bleeding) admitted to our department between January and December 2001 were included in this prospective, case-control study if they were current users of NSAIDs, including aspirin (defined as use of at least one dose within the last 7 days before the episode of bleeding), and had endoscopically verified gastric or duodenal ulcer or hemorrhagic/erosive gastritis as the most probable source of bleeding. All endoscopies were performed within 24 hours after admission. Controls were recruited from the outpatient medicine clinics. They were current (within the last 7 days) users of NSAIDs, including aspirin, without signs of gastrointestinal bleeding. Controls were matched to patients at a 1:1 ratio by sex and by age in 5-year bands (17). All subjects gave informed consent to participate in the study. There were no exclusion criteria, but 3 NSAID users admitted with upper gastrointestinal bleeding were excluded: 2 with uncertain sources of bleeding and 1 who died within a few hours of admission.

A structured questionnaire was completed for all cases and controls. The duration of NSAID/aspirin use was considered to be short if the drug had been taken for ≤3 months. NSAID dose was expressed as the number of defined daily doses (18) consumed within the last 7 days.

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For example, one defined daily dose of aspirin was considered to be 150 mg, whereas low-dose aspirin was considered to be the consumption of seven or fewer defined daily doses (1050 mg within the last 7 days).

H. pylori Testing

H. pylori infection was diagnosed by serum immunoglobulin (Ig) G antibodies or the ^{13}C-urea breath test. IgG antibodies against *H. pylori* were measured in duplicate by an in-house enzyme immunoassay (19) using rabbit anti-human IgG (IDEIA *Helicobacter pylori* IgG; Dako, Copenhagen, Denmark). Antibodies against *H. pylori* were determined in cases on the day after admission, and in controls on the day of completion of the study questionnaire. After at least an 8-hour fasting period, ^{13}C-urea breath tests were performed after the patients drank 75 mg of ^{13}C-labeled urea dissolved in 50 mL of water and 200 mL of orange juice. Duplicate breath samples were taken before and 30 minutes after the ingestion of urea and analyzed by infrared spectroscopy. The breath test was considered to be positive for *H. pylori* if the increase in the $^{13}C/^{12}C$ ratio was >4‰. A ^{13}C-urea breath test was performed in 65 of the cases as soon as oral intake was allowed, as well as in 51 controls. *H. pylori* status was considered to be positive if either the serological test or the urea breath test was positive.

CagA status was determined by serum antibodies against CagA protein using a commercially available enzyme immunoassay (*Helicobacter pylori* CagA IgG EIA WELL; Radim, Liege, Belgium) according to the manufacturer's instructions.

Statistical Analysis

All data were analyzed using SPSS, version 10.1 (SPSS Inc., Chicago, Illinois). Proportions were compared with the chi-squared test or Fisher exact test. Continuous variables were compared with the Student *t* test. Multivariate analysis was performed using logistic regression models. Variables associated with the dependent variable (at $P \leq 0.10$) were entered in the multivariate models. A two-tailed P value <0.05 was considered to be significant.

RESULTS

We enrolled 80 cases of bleeding and 80 controls. Age, sex, and mean dose of NSAID used were similar between cases and controls (Table 1), but cases were more likely to have used NSAIDs for <3 months (46 [58%] cases vs. 32 [40%] controls, $P = 0.04$). Aspirin was the most common NSAID used within the last 7 days in both cases and controls (Table 2).

Upper gastrointestinal bleeding was associated with gastric ulcer(s) only in 24 patients (30%), duodenal ulcer only in 21 patients (26%), both gastric and duodenal ulcers in 12 patients (15%), hemorrhagic gastritis only in 8 patients (10%), gastric ulcer and hemorrhagic gastritis in 6 patients (8%), duodenal ulcer and hemorrhagic gastritis in 6 patients (8%), and gastric and duodenal ulcers and hemorrhagic gastritis in 3 patients (4%).

A previous history of peptic ulcer disease, upper gastrointestinal bleeding, and perhaps dyspepsia within the last 2 weeks were more common in cases than controls (Table 1). In contrast, there was no difference between the two groups in smoking habits, alcohol consumption, recent use of corticosteroids, use of anticoagulants, or comorbid diseases (Table 1).

Association of H. pylori Infection with Bleeding

H. pylori infection was detected in 63 cases (79%) and 45 controls (56%) ($P = 0.004$; Table 1). Two cases and 1 control had discordant results between the serologic test and the ^{13}C-urea breath test; exclusion of these patients did not affect the results. Similarly, among patients who underwent the ^{13}C-urea breath test, *H. pylori* infection was present in 78% (51/65) of the cases of bleeding and 57% (29/51) of the controls ($P = 0.02$).

There were no significant differences in CagA positivity either between the cases and controls, or between the *H. pylori*–positive cases and controls (Table 1).

In a multivariate logistic regression analysis, *H. pylori* infection was the only significant risk factor for upper gastrointestinal bleeding (odds ratio = 1.7; 95% confidence interval: 1.2 to 2.5; $P = 0.004$), although both a history of peptic ulcer disease and a short duration of NSAID use were more common in cases (Table 3). An analysis to investigate whether the effect of *H. pylori* on the risk of bleeding differed by the type of NSAID (aspirin vs. other) found no evidence of a statistically significant interaction ($P = 0.22$).

DISCUSSION

We found that *H. pylori* infection was an independent risk factor for upper gastrointestinal bleeding in users of NSAIDs, including aspirin, consistent with the results of a recent meta-analysis (20). In our study, *H. pylori* infection almost doubled the risk of upper gastrointestinal bleeding among NSAID users after adjustment for other risk factors for bleeding. Although the reported effects of *H. pylori* infection on the risk of upper gastrointestinal bleeding vary widely, from a 50% decrease (13,21) to a threefold increase (22), differences in the inclusion criteria for cases of bleeding and for controls may be responsible for many of the discrepancies (12,13,23,24). Our sample consisted exclusively of current NSAID users and our findings are in agreement with those from studies with similar design and inclusion criteria (11,25).

Table 1. Characteristics of Cases of Gastrointestinal Bleeding and Controls*

Characteristic	Cases of Bleeding (n = 80)	Controls (n = 80)	*P* Value
	Number (%) or Mean ± SD		
Age (years)	67 ± 15	66 ± 15	0.63
Male sex	53 (66)	52 (65)	1.00
History of peptic ulcer	27 (34)	10 (13)	0.003
History of upper gastrointestinal bleeding	15 (19)	5 (6)	0.03
Dyspepsia within the last 2 weeks	23 (29)	12 (15)	0.06
Current smoking	18 (23)	17 (21)	1.00
Alcohol >20 g/wk	17 (21)	9 (11)	0.13
Type of NSAID			0.21
Nonaspirin NSAID	28 (35)	26 (33)	
Aspirin	47 (59)	53 (66)	
Both	5 (6)	1 (1)	
NSAID duration			0.16
<1 week	21 (26)	16 (20)	
1 week–3 months	25 (31)	16 (20)	
3–12 months	12 (15)	19 (24)	
>12 months	22 (28)	29 (36)	
Dose of NSAID			0.15
<3.5 defined daily doses/wk	18 (23)	9 (11)	
3.5–7 defined daily doses/wk	33 (41)	40 (50)	
>7 defined daily doses/wk	29 (36)	31 (39)	
Corticosteroids	3 (4)	3 (4)	1.00
Anticoagulants	2 (3)	1 (1)	1.00
Other drugs	57 (71)	60 (75)	0.72
Comorbid diseases			0.15
None/other	35 (44)	29 (36)	
Heart disease	30 (38)	39 (49)	
Cerebrovascular disease	5 (6)	3 (4)	
Hypertension	9 (11)	4 (5)	
Rheumatoid arthritis	1 (1)	5 (6)	
Helicobacter pylori infection	63 (79)	45 (56)	0.004
CagA seropositivity	48 (60)	37 (46)	0.11
CagA seropositivity in *H. pylori*-positive cases, n/N (%)	48/63 (76)	37/45 (82)	0.61

* All cases and controls were users of NSAIDs.
CagA = cytotoxin-associated antigen; NSAID = nonsteroidal anti-inflammatory drug.

CagA status of *H. pylori* did not affect the risk of upper gastrointestinal bleeding among NSAID users in our study. More than 75% of *H. pylori*–positive cases and controls were CagA positive. Although CagA seropositivity has been associated with a higher risk of ulcer bleeding (21), it was not associated with the risk of bleeding in a study that included low-dose aspirin users (26).

We found no differences between aspirin and other NSAIDs in terms of their effects on the risk of upper gastrointestinal bleeding, as has been seen in other case-control studies (11,25). However, the results of a recent randomized trial suggested that the effect of *H. pylori* infection on the risk of bleeding may differ between users of aspirin and other NSAIDs (27).

Short duration (≤3 months) of NSAID use was more common in cases of bleeding than in controls, consistent with observations that the risk of gastrointestinal complications is higher during the first months of NSAID use (1,28). In addition, previous peptic ulcer disease or upper gastrointestinal bleeding, and recent dyspepsia, were more common among cases (17,26,28,29).

In conclusion, *H. pylori* infection almost doubled the risk of upper gastrointestinal bleeding among users of NSAIDs, whereas CagA status was not associated with the risk of bleeding. Thus, as has been suggested by others (11,25,27), *H. pylori* eradication may reduce the risk of upper gastrointestinal bleeding among NSAID users. Whether *H. pylori* eradication alone is sufficient, or

Table 2. Type of Nonsteroidal Anti-inflammatory Drugs Used by Cases (within the 7 Days before the Bleeding Episode) and by Controls (within the 7 Days before Completion of Questionnaire)

Type of NSAID	Cases of Bleeding (n = 80)	Controls (n = 80)
	Number	
Nonaspirin NSAID	28	26
Aceclofenac	2*	5
Diclofenac	4*†‡	6
Ibuprofen	1	1
Indomethacin	1	0
Ketoprofen	2	0
Mefenamic acid	2	3‖
Meloxicam	3§	0
Naproxen	2	2
Niflumol	1	0
Nimesulid	10*†	11‖
Piroxicam	3	0
Tenoxicam	2§	0
Aspirin	47	53
Aspirin	25	13
Salospir	21	39
Egicalm	1	1
Nonaspirin NSAID + aspirin	5	1

* Both aceclofenac and nimesulid were used by 1 case of bleeding.
† Both diclofenac and nimesulid were used by 2 cases of bleeding.
‡ Both diclofenac and meloxicam were used by 1 case of bleeding.
§ Both diclofenac and tenoxicam were used by 1 case of bleeding.
‖ Both mefenamic acid and nimesulid were used by 2 controls.
NSAID = nonsteroidal anti-inflammatory drug.

whether additional long-term use of antisecretory drugs should be advised in high-risk subgroups of NSAID users (30), should be evaluated in prospective clinical trials.

Table 3. Factors Associated with Upper Gastrointestinal Bleeding in a Multivariate Analysis

Factor	Odds Ratio (95% Confidence Interval)*	*P* Value
Helicobacter pylori infection	1.7 (1.2–2.5)	0.004
History of peptic ulcer	1.8 (0.97–3.2)	0.06
History of upper gastrointestinal bleeding	1.2 (0.5–2.5)	0.73
Dyspepsia within the last 2 weeks	1.4 (0.9–2.1)	0.15
NSAID duration of use ≤3 months†	1.4 (0.9–2.1)	0.10

* Also adjusted for the type of NSAID used (nonaspirin only, aspirin only, or both).
† Compared with >3 months.
NSAID = nonsteroidal anti-inflammatory drug.

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P.O.Box 2345, Beijing 100023,China
Fax: +86-10-6528 1093
E-mail: wjg@public.bta.net.cn www.wjgnet.com

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• REVIEW •

Helicobacter pylori infection and respiratory diseases: a review

Anastasios Roussos, Nikiforos Philippou, Konstantinos I Gourgoulianis

Anastasios Roussos, Nikiforos Philippou, 9th Department of Pulmonary Medicine, "SOTIRIA" Chest Diseases Hospital, Athens, Greece
Konstantinos I Gourgoulianis, Pulmonary Department, Medical University of Thessaly, Larisa, Greece
Correspondence to: Dr Anastasios Roussos, 20 Ierosolimon Street, PO: 11252, Athens, Greece. roumar26@yahoo.com
Telephone: +301-8646215 **Fax:** +301-8646215
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Abstract

In the past few years, a variety of extradigestive disorders, including cardiovascular, skin, rheumatic and liver diseases, have been associated with *Helicobacter pylori* (*H. pylori*) infection. The activation of inflammatory mediators by *H. pylori* seems to be the pathogenetic mechanism underlying the observed associations. The present review summarizes the current literature, including our own studies, concerning the association between *H. pylori* infection and respiratory diseases.

A small number of epidemiological and serologic, case-control studies suggest that *H. pylori* infection may be associated with the development of chronic bronchitis. A frequent coexistence of pulmonary tuberculosis and *H. pylori* infection has also been found. Moreover, recent studies have shown an increased *H. pylori* seroprevalence in patients with bronchiectasis and in those with lung cancer. On the other hand, bronchial asthma seems not to be related with *H. pylori* infection.

All associations between *H. pylori* infection and respiratory diseases are primarily based on case-control studies, concerning relatively small numbers of patients. Moreover, there is a lack of studies focused on the pathogenetic link between respiratory diseases and *H. pylori* infection. Therefore, we believe that larger studies should be undertaken to confirm the observed results and to clarify the underlying pathogenetic mechanisms.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a slow-growing, microaerophilic, gram-negative bacterium, whose most striking biochemical characteristic is the abundant production of urease. This bacterium colonizes gastric mucosa and elicits both inflammatory and immune lifelong responses, with release of various bacterial and host-dependent cytotoxic substances[1]. Pathological studies and extensive clinical trials, carried out in the past few years, have proved the causative role of *H. pylori* in the development of chronic gastritis[2] and peptic ulcer disease[3]. It seems that this bacterium is also causally related to low-grade B-cell lymphoma of gastric mucosa-associated-lymphoid-tissue (MALT-lymphoma)[4]. Moreover, *H. pylori* infection has been established as a risk factor for the development of both diffuse and intestinal types of gastric cancer[5].

Recent studies suggest an epidemiological association between *H. pylori* infection and several extragastroduodenal pathologies, including cardiovascular, skin, rheumatic and liver diseases (Table 1)[6,7]. Unfortunately, such epidemiological studies are influenced by a wide variety of confounding factors, i.e. socioeconomic status, time of acquisition of the infection, presence of different bacterial strains and previous antibiotic therapy. However, according to many authors, the observed associations might be true and explained by a role of *H. pylori* infection in the pathogenesis of certain extradigestive disorders. It is well known that *H. pylori* colonization of the gastric mucosa stimulates the release of various proinflammatory substances, such as cytokines, eicosanoids and proteins of the acute phase[8]. Moreover, a cross mimicry between bacterial and host antigens exists in *H. pylori* infected patients[9]. Therefore, a pathogenetic link between *H. pylori* infection and diseases characterized by activation of inflammatory mediators and/or induction of autoimmunity might exist.

Chronic inflammation and increased immune response have been observed in a variety of respiratory diseases, including chronic bronchitis[10,11] and bronchiectasis[12]. Moreover, both chronic obstructive pulmonary disease[13,14] and pulmonary tuberculosis[15] are more prevalent in peptic ulcer patients than in the general population. Based on these facts, many recent studies have focused on the potential association between *H. pylori* infection and various respiratory disorders. Table 2 summarizes those respiratory diseases whose relation with *H. pylori* infection has been studied in the literature.

The aim of the present report is to provide a critical review of the current literature, including our own studies, as regards the association between *H. pylori* infection and respiratory diseases.

Table 1[6,7] Extradigestive diseases associated with *H. pylori* infection

Vascular diseases
Ischaemic heart disease
Primary Raynaud' s phenomenon
Primary headache
Skin diseases
Idiopathic chronic urticaria
Rosacea
Alopecia areata
Autoimmune diseases
Sjogren' s syndrome
Autoimmune thyroiditis
Autoimmune thrombocytopenia
Schoenlein-Henoch purpura
Other diseases
Liver cirrhosis
Growth retardation
Chronic idiopathic sideropenia
Sudden infant death
Diabetes mellitus

Table 2 Respiratory diseases studied for a relationship with *H. pylori* infection

Chronic bronchitis
Pulmonary tuberculosis
Bronchiectasis
Lung cancer
Bronchial asthma

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HELICOBACTER PYLORI INFECTION AND CHRONIC BRONCHITIS

Chronic bronchitis is a pulmonary disease characterized by, primarily irreversible, airflow obstruction due to the chronic inflammation of the small airways. The presence of airflow obstruction that is not fully reversible is confirmed by spirometry (postbronchodilator FEV1<80 % of the predicted value, in combination with an FEV1/FVC<70 %). Although its true prevalence remains unknown, it is estimated that approximately 12.5 million persons in the United States suffer from chronic bronchitis[14].

Chronic bronchitis 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 chronic bronchitis in peptic ulcer patients was increased two-to-three fold compared with that in ulcer-free controls[12,14,15]. Moreover, a follow-up study demonstrated that chronic bronchitis was a major cause of death among patients with peptic ulcer disease[16].

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 chronic bronchitis. However, in 1998, Gaseli and colleagues carried out a prospective pilot study in a sample of 60 Italian patients with chronic bronchitis and found an increased seroprevalence of *H. pylori* infection compared to that detected in 69 healthy controls (81.6 % versus 57.9 % respectively, P=0.008). In this study, the odds ratio for chronic bronchitis in the presence of *H. pylori* infection, calculated after adjustment for age and social status, was 3.4[10]. These results suggested, for the first time, that *H. pylori* infection per se might be related to an increased risk of developing chronic bronchitis. Two years later, a large epidemiological study in 3608 Danish adults showed that chronic bronchitis might be more prevalent in *H. pylori* IgG seropositive women than in uninfected ones (odds ratio 1.6, with a 95 % confidence interval of 1.1-2.5)[20]. In order to further investigate the reported association between *H. pylori* infection and chronic bronchitis, we recently performed a case-control study in a cohort of 144 Greek patients with chronic bronchitis and 120 control subjects. Our results were in accordance with those of Gaselli and associates, as we also found that *H. pylori* seropositivity in patients was significantly higher than that in controls (83.3 % *vs* 60 %, P=0.007)[21].

The mechanisms underlying the suggested association between *H. pylori* infection and chronic bronchitis remain unclear. This association might reflect either susceptibility induced by common factors or a kind of causal relationship between these two conditions. It is well known that age, sex and socioeconomic status are related with both *H. pylori* infection[21] and risk of developing chronic bronchitis[14]. However, in all mentioned studies above patients with chronic bronchitis were well matched with control subjects for these parameters. Tobacco use could be another confounding factor. Cigarette smoking is the major cause of chronic bronchitis[9]. On the other hand, data on the relation between *H. pylori* infection and smoking habits are controversial. The prevalence of *H. pylori* infection in smokers has been variously reported as low[22], normal[23], and high[24]. As the relation between smoking and *H. pylori* infection has not been clarified yet, the possible impact of cigarette smoking on both chronic bronchitis and *H. pylori* infection should be regarded as a potential limitation of the reviewed studies.

Unfortunately, there are no studies in the literature focused on the potential aetio-pathogenetic role of *H. pylori* infection in chronic bronchitis. Some authors hypothesized that the chronic activation of inflammatory mediators induced by *H. pylori* infection might lead to the development of a non-specific inflammatory process, such as chronic bronchitis[19,25]. It is well known that *H. pylori* and particularly those strains bearing the cytotoxin associated gene-A (cagA positive strains), stimulates the release of a variety of proinflammatory cytokines, including interleukin-1 (IL-1), IL-8 and tumour necrosis factor-alpha[27,28]. The eradication of *H. pylori* leads to normalization of serum cytokines levels[17]. Recent studies showed that the same cytokines might be released during the course and exacerbations of chronic bronchitis[9,11,29]. The underlying mechanisms, which induce and control this inflammatory process in chronic bronchitis, are still unclear. Therefore, we could hypothesize that *H. pylori* infection might play a proinflammatory role and co-trigger chronic bronchitis with other more specific environmental, genetic and unknown yet factors.

In conclusion, the primary evidence for an association between *H. pylori* infection and chronic bronchitis rests on serologic, case-control studies. Studies estimating the relative risk of developing chronic bronchitis for *H. pylori* infected patients and the effect of *H. pylori* eradication on the natural history of chronic bronchitis should be undertaken. The pathogenetic mechanisms underlying this association need also further evaluation. Future studies concerning this aspect should be focused on the prevalence of cagA positive Helicobacter strains and their induced proinflammatory markers, in patients with chronic bronchitis.

HELICOBACTER PYLORI INFECTION AND PULMONARY TUBERCULOSIS

Tuberculosis (TB) is a chronic bacterial infection caused by Mycobacterium tuberculosis and characterized by the formation of granulomas in infected tissues and by cell-mediated hypersensitivity. The lungs are primarily infected. However, any other organ may be involved. Although there is a lack of epidemiological evidence concerning the worldwide prevalence of TB, it has been estimated that one third of the world population is infected with Mycobacterium tuberculosis and there are ten million new cases of active TB each year. The vast majority of them occur in the developing countries, where TB remains a common health problem[30].

In 1992, Mitchell *et al* carried out a large cross-sectional study concerning the *H. pylori* epidemiology in a southern China population. They found that a history of pulmonary TB might be associated with an increased prevalence of *H. pylori* infection[30]. More recently, Woeltje *et al* assessed the prevalence of tuberculin skin test (TST) positivity in a cohort of 346 newly hospitalized patients. A history of peptic ulcer disease was one of the identified risk factors for a positive TST test (odds ratio: 4.53, P=0.017)[31]. In order to further investigate the possible association between pulmonary TB and *H. pylori* infection, Sanaka *et al* performed, in 1998, a serologic case-control study in a hospitalized population. No difference in *H. pylori* seroprevalence among 40 inpatients on antituberculosis chemotherapy for less than three months, 43 TB patients on chemotherapy for more than three months and 60 control subjects was detected (73.3 %, 65 % and 69.8 % respectively, P>0.5 in all comparisons)[32]. However, in this study the eradication of *H. pylori* by antituberculosis drugs could not be excluded. Rifampicin and Streptomycin, two drugs commonly used in antituberculosis regimens, are effective against *H. pylori* and decrease in *H. pylori* seroprevalence during antituberculosis therapy has been reported[33,34]. Therefore, we recently carried out a case-control study focused on the seroprevalence of *H. pylori* in TB patients, before the initiation of antituberculosis treatment. A total of 80 TB patients and 70 control subjects, well matched for age, sex and social

status, were recruited into this study. We found that the *H. pylori* seropositivity in the TB group was significantly higher than that of controls (87.5 % *vs* 61.4 %, P=0.02). The mean serum concentration of IgG antibodies against *H. pylori* was also significantly higher in TB patients than in control subjects (39.0±25.2 U/ml *vs* 26.1±21.2 U/ml, P=0.001)[37].

Taken together, data in the literature on the relationship between *H. pylori* infection and pulmonary TB are still insufficient. The observed frequent coexistence of both infections must be confirmed in a larger number of patients. This coexistence might reflect susceptibility to both *H. pylori* and Mycobacterium tuberculosis induced by common host genetic factors. It has been suggested that HLA-DQ serotype may contribute to enhanced mycobacterial survival and replication[36]. Recent studies showed that the same serotype is also associated with increased susceptibility to *H. pylori* infection[37,38]. Poor socioeconomic and sanitary conditions during childhood could be another factor responsible for the association between the two infections, as it is well known that in developing countries acquisition of both *H. pylori* and Mycobacterium tuberculosis occurs early in life[39,40]. Therefore, we believe that studies focused on the common, either genetic or environmental, predisposition to both bacteria are needed.

HELICOBACTER PYLORI INFECTION AND BRONCHIECTASIS

Bronchiectasis is an abnormal and permanent dilation of bronchi, due to inflammation and destruction of the structural components of the bronchial wall. Persistent or recurrent cough, purulent sputum production and/or hemoptysis are symptoms presented during the clinical course of this disorder. A wide variety of respiratory infections, toxic substances and rare congenital syndromes are associated with the development of bronchiectasis. However, a great percentage of cases are of unknown cause[41].

In 1998, Tsang *et al* found that the *H. pylori* seroprevalence in 100 patients with bronchiectasis (76 %) was higher than that in the controls (54.3 %, P=0.001). Further analysis in studied patients revealed an association between *H. pylori* seropositivity and 24-hours sputum volume (P=0.03)[42].

As far as we know, the study of Tsang *et al* is the only report in the literature concerning the association between *H. pylori* infection and bronchiectasis. The authors hypothesized that the spilling or inhalation of *H. pylori* into the respiratory tract might lead to a chronic bronchial inflammatory disorder such as bronchiectasis. However, although *H. pylori* has been identified in the tracheobronchial aspirates in mechanically ventilated patients[43], neither identification in human bronchial tissue nor isolation from bronchoalveolar lavage (BAL) fluid have been achieved yet[1]. On the other hand, recent studies have shown that inflammation in bronchiectasis is primarily cytokine-mediated[42,44]. Therefore, the activation of systemic inflammatory mediators by chronic *H. pylori* infection could represent a possible pathogenetic link between these two diseases.

In conclusion, the possible association between *H. pylori* and bronchiectasis seems intriguing and might have a pathogenetic basis. However, studies in larger series are needed to confirm this association and to clarify the underlying mechanisms. As pulmonary TB is a common cause of bronchiectasis, we believe that the increased prevalence of *H. pylori* infection in TB patients should be taken into account in the design of these future studies.

HELICOBACTER PYLORI INFECTION AND OTHER RESPIRATORY DISEASES

Lung cancer A recent study showed a higher *H. pylori* seroprevalence (89.5 %) among 50 patients with lung cancer than that in control subjects (64 %, P<0.05). The CagA strain seropositivity was about thrice as high as in controls. (63 % *vs* 21.5 % respectively, P<0.05). Lung cancer patients were characterized by a significant increase of gastrin concentration in both serum and bronchoalveolar lavage (BAL). An enhanced m-RNA expression for gastrin and its receptor, as well as for cyclooxygenase (COX)-2, in the tumor tissue was also detected. Therefore, the authors hypothesized that *H. pylori* might contribute to lung carcinogenesis, via enhancement of gastrin synthesis. Gastrin might induce increased mucosal cell proliferation of bronchial epithelium and lead to atrophy and induction of COX-2, as it happens in gastric cancer. Finally, the authors proposed that *H. pylori* should be eradicated in lung cancer patients, in order to reduce the *H. pylori* provoked hypergastrinemia and COX-2 expression[45].

Chronic bronchitis, which is associated with both lung cancer and *H. pylori* infection, might be a confounding factor in this study. Moreover, although some authors have also showed an increased gastrin concentration in serum and BAL fluid in lung cancer patients[46,47], others did not confirm this finding[48]. Therefore, we believe that before adapting the *H. pylori* eradication in lung cancer patients, further studies are needed to examine whether the reported epidemiological association between these two diseases has a pathogenetic basis.

Bronchial asthma In 2000, Tsang *et al* estimated the prevalence of *H. pylori* infection in a cohort of 90 patients with bronchial asthma. *Helicobacter pylori* seroprevalence did not differ significantly between asthmatic and control subjects (47.3 % *vs* 38.1 %, P>0.05), while serum concentration of IgG antibodies against *H. pylori* did not correlate with spirometric values and duration of asthma. The authors concluded that bronchial asthma might not be associated with *H. pylori* infection[49]. Moreover, as far as we know there is a lack of a theoretical hypothesis that might explain a possible association between these two diseases. Therefore, we believe that our knowledge on the association between *H. pylori* infection and respiratory diseases is unlikely to be advanced by more studies concerning the prevalence of *H. pylori* infection in patients with bronchial asthma.

CONCLUSIONS-FUTURE CHALLENGES

At present, the primary evidence for a link between *H. pylori* infection and respiratory diseases rests on case-control studies, concerning relatively small numbers of patients. Future studies should be large enough for moderate-sized effects to be assessed or registered reliably. The activation of inflammatory mediators by *H. pylori* infection might be the pathogenetic mechanism underlying the observed associations. Therefore, the role of genetic predisposition of the infected host, the presence of strain-specific virulence factors and the serum concentration of proinflammatory markers in *H. pylori* infected patients with respiratory diseases needs further evaluation. Finally, randomized control studies should be undertaken, in order to clarify the effect of the *H. pylori* eradication on the prevention, development and natural history of these disorders.

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In Vitro and In Vivo Inhibition of *Helicobacter pylori* by *Lactobacillus casei* Strain Shirota

D. Sgouras,[1]† P. Maragkoudakis,[2]† K. Petraki,[3]† B. Martinez-Gonzalez,[1] E. Eriotou,[1] S. Michopoulos,[4] G. Kalantzopoulos,[2] E. Tsakalidou,[2] and A. Mentis[1]*

Laboratory of Medical Microbiology, Hellenic Pasteur Institute,[1] Laboratory of Dairy Research, Agricultural University of Athens,[2] Laboratory of Pathology, Hippocration Hospital,[3] and Gastroenterology Clinic, Alexandra Hospital,[4] Athens, Greece

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We studied the potential inhibitory effect of *Lactobacillus casei* strain Shirota (from the fermented milk product Yakult [Yakult Ltd., Tokyo, Japan]) on *Helicobacter pylori* by using (i) in vitro inhibition assays with *H. pylori* SS1 (Sydney strain 1) and nine *H. pylori* clinical isolates and (ii) the in vivo *H. pylori* SS1 mouse model of infection over a period of 9 months. In vitro activity against *H. pylori* SS1 and all of the clinical isolates was observed in the presence of viable *L. casei* strain Shirota cells but not in the cell-free culture supernatant, although there was profound inhibition of urease activity. In vivo experiments were performed by oral administration of *L. casei* strain Shirota in the water supply over a period of 9 months to 6-week-old C57BL/6 mice previously infected with *H. pylori* SS1 (study group; *n* = 25). Appropriate control groups of *H. pylori*-infected but untreated animals (*n* = 25) and uninfected animals given *L. casei* strain Shirota (*n* = 25) also were included in the study. *H. pylori* colonization and development of gastritis were assessed at 1, 2, 3, 6, and 9 months postinfection. A significant reduction in the levels of *H. pylori* colonization was observed in the antrum and body mucosa in vivo in the lactobacillus-treated study group, as assessed by viable cultures, compared to the levels in the *H. pylori*-infected control group. This reduction was accompanied by a significant decline in the associated chronic and active gastric mucosal inflammation observed at each time point throughout the observation period. A trend toward a decrease in the anti-*H. pylori* immunoglobulin G response was measured in the serum of the animals treated with lactobacillus, although this decrease was not significant.

Lactic acid bacteria are widely used in the production of fermented foods and beverages and contribute both to the sensory qualities of the food and to the prevention of spoilage. Moreover, they are present in large numbers in the normal human and animal gastrointestinal floras. In recent decades, much attention has been paid to the health-promoting (probiotic) properties of lactobacilli, as it has been claimed that when administered in adequate amounts, they confer a health benefit on the host (Joint FAO/W. H. O. 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). Furthermore, for use in foods, probiotic microorganisms not only should be capable of surviving passage through the digestive tract but also should have the capability to proliferate in the gut (Joint FAO/W. H. O. Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria). Several probiotic lactobacilli have been shown to survive transit through the human gastrointestinal tract and to maintain a balanced intestinal microflora (32). In particular, *Lactobacillus casei* strain Shirota, contained in the commercial fermented milk product Yakult (Yakult Ltd., Tokyo, Japan), has been reported to exhibit antitumor, immunostimulatory, and antimicrobial activities. More specifically, the oral administration of *L. casei* strain Shirota stimulated a type 1 response (Th1), activated the cellular immune system, and inhibited the incidence of tumors and immunoglobulin E (IgE) production in a murine model (22, 34). Furthermore, *L. casei* strain Shirota orally administered to rats infected with *Listeria monocytogenes* enhanced cellular immunity, as determined by a delayed-type hypersensitivity reaction, thus conferring enhanced resistance against the pathogen (8). Finally, *L. casei* strain Shirota administration before or after an initial challenge dramatically inhibited *Escherichia coli* growth in a murine model of urinary tract infection and decreased the severity of diarrhea resulting from Shiga toxin-producing *E. coli* O157:H7 in infant rabbits (3, 25).

Helicobacter pylori, a spiral gram-negative microaerophilic stomach pathogen that infects over 50% of the population worldwide (10), is the cause of gastritis (4) and peptic ulcers (11) and has been linked to the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (27). Recently, attention has been paid to the interactions between *H. pylori* and probiotic lactobacilli. *H. pylori* colonization was inhibited in *Lactobacillus salivarius*-fed gnotobiotic BALB/c mice, and *H. pylori*-specific antibody titers became marginal, while *H. pylori* colonized in large numbers and caused active gastritis in lactobacillus-free mice (15). In another study, the oral administration of a spent culture supernatant of *L. acidophilus* resulted in the suppression of *H. felis* in a murine infection model (6, 7). In addition, an *L. acidophilus* culture supernatant was effective in vitro and had a partial, long-term suppressive effect on *H. pylori* in humans (24). Finally, in a recent intervention study involving administration of the fermented milk product Yakult containing *L. casei* strain

* Corresponding author. Mailing address: Laboratory of Medical Microbiology, Hellenic Pasteur Institute, Vas. Sophias 127, 115 21 Athens, Greece. Phone: 30210-6478816. Fax: 30210-6423488. E-mail: mentis@mail.pasteur.gr.

† D.S., P.M., and K.P. contributed equally to this work.

Analysis of the results from the in vivo experiments was performed with respect to *H. pylori* colonization by the Mann-Whitney test (GraphPad) and with respect to the associated gastritis by the Wilcoxon rank sum test due to the ordinal nature of the data (14). Finally, anti-*H. pylori* IgG antibodies were detected in the collected serum samples by an in-house enzyme-linked immunosorbent assay (ELISA). Briefly, 15 μg of *H. pylori* SS1 antigen produced by sonication and subsequent dialysis (SpectraPor; cutoff pore size, 8 kDa) was used to coat 96-well plates. Collected mouse serum samples (diluted 1:50) were incubated on the plates for 24 h at 4°C (primary incubation), and then rabbit anti-mouse IgG (entire molecule)– peroxidase conjugate (Sigma) was used for the secondary incubation (2 h at 37°C). Color was developed by the addition of *o*-phenylenediamine (Sigma), and the optical density at 492 nm was measured with a Sunrise microtiter plate reader.

Bacterial detection in vivo. *H. pylori* in gastric tissue was detected by quantitative culturing, *H. pylori*-specific PCR, and histopathologic evaluation. For *H. pylori* SS1 quantitative culturing, preweighed half-stomach samples were homogenized in thioglycolate medium (Oxoid), serially diluted in phosphate-buffered saline, and plated on CHW agar plates with antibiotics. The cultures were incubated under microaerophilic conditions at 37°C for up to 6 days. *H. pylori* colonies were visualized on the basis of urease activity (12), and results were expressed as CFU per gram of gastric tissue. For *L. casei* strain Shirota isolation, stomach, intestinal, and fecal samples prepared as described above were plated on MRS agar supplemented with vancomycin (10 μg/ml) and polymyxin B (10 μg/ml) and incubated at 30°C for 48 to 72 h. Colonies were characterized on the basis of morphology, Gram stain, sugar fermentation patterns (API 50CHL kit; BioMérieux, Marcy l'Etoile, France), and species-specific PCR.

PCR conditions. Genomic DNA for the detection by PCR of bacterial strains in tissue samples or bacterial colonies was isolated by the standard phenol-chloroform method (28). For the detection of *H. pylori* by PCR, specific primers for the *ureC* (*glmM*) gene were used (19). For *L. casei* identification, species-specific PCR was performed with primers Prl and CasII for the 16S-23S intergenic spacer region (33).

Histopathologic analysis of gastric tissue samples. Excised stomachs were opened along the lesser curvature, and the longitudinal half was fixed in 10% neutral buffered formalin solution, embedded in paraffin, and processed for histopathologic analysis. Antral, body, and cardioesophageal mucosa samples were examined in the same section. Eleven serial longitudinal 4-μm sections were cut from each specimen; 9 of them were stained with hematoxylin-eosin (H&E) for evaluation of gastric inflammation, and 2 were stained by the May-Grünwald Giemsa stain method for the assessment of *H. pylori* colonization. The bacterial density was scored from 0 to 4, according to the Lausanne criteria (17). The pathologic characteristics of the gastric mucosa were assessed according to the updated Sydney system (9). Histopathologic evaluation was performed with no prior knowledge of the identity of the samples by the histopathologist.

RESULTS

In vitro screening for *H. pylori* inhibition. With the well diffusion assay, the activity of *L. casei* strain Shirota against *H. pylori* SS1 and wild-type clinical isolates ($n = 9$) was assessed. Antimicrobial activity was evident only when *L. casei* strain Shirota cells from o/n cultures (inhibition zones, 12.7 to 15.7 mm) or cells washed and resuspended in fresh MRS medium (inhibition zones, 11.0 to 15.0 mm) were used. No significant difference in anti-*H. pylori* activity was observed between o/n cultures of *L. casei* strain Shirota and cells washed and resuspended in MRS medium. Likewise, no inhibition was observed when the *L. casei* strain Shirota cell-free culture supernatant was used at its native pH 4.5, or at pH 6.5 or when *E. coli* HB101 was used instead of *L. casei* strain Shirota. These results suggest that the presence of living *L. casei* strain Shirota is required for *H. pylori* inhibition.

To elaborate further on the mechanism of in vitro *H. pylori* inhibition, we studied the effect of the *L. casei* strain Shirota supernatant on urease activity and simultaneously measured the effect on *H. pylori* viability in liquid *H. pylori* cultures. The *L. casei* strain Shirota supernatant was tested only at 10% the final incubation volume, because initial experiments involving higher percentages in MRS medium resulted in reduced viability of *H. pylori*. In the presence of 10% *L. casei* strain Shirota cell-free supernatant at pH 4.5, inhibition of *H. pylori* urease activity (Fig. 1a) and viability (Fig. 1b) was observed. Similar results were obtained with the respective pH 4.5 MRS medium control.

In the above-described experiments, the pH of the incubation medium was 5.1 ± 0.2 due to the acidic pH (4.5) of the *L. casei* strain Shirota supernatant or acidified MRS medium. The concentration of lactic acid in the *L. casei* strain Shirota supernatant-containing incubation medium, determined by HPLC, was about 15 mM. To exclude the effect of a low pH on *H. pylori* urease activity and viability, an *L. casei* strain Shirota cell-free supernatant at pH 6.5, adjusted by the addition of NaOH, also was used. Urease activity was strongly inhibited in these samples (Fig. 1a), although no inhibition of *H. pylori* growth was observed (Fig. 1b). In the same experiments, the presence of a 10% pH 6.5 MRS medium control did not affect the urease activity or viability of *H. pylori* cells.

Inclusion of amoxicillin at 0.12 μg/ml (two times the MIC) as a positive control for *H. pylori* growth inhibition (Fig. 1b) resulted in a concomitant reduction in urease activity (Fig. 1a). Finally, in experiments in which *H. pylori* was incubated for 24 h with DL-lactic acid (at 100, 50, 25, 10, 5, 1, and 0 mM concentrations), over 70% inhibition of urease activity was seen at concentrations of lactic acid higher than 15 mM at 3 and 24 h (data not shown).

Evaluation of in vivo *H. pylori* colonization and associated gastritis in the infected and uninfected control groups. The presence of *H. pylori* and associated gastritis was not observed in any of the uninfected control animals. However, *H. pylori* was successfully detected in the gastric mucosa of mice in the *H. pylori* SS1 group throughout the entire 9-month observation period by viable counts, PCR, and histopathologic evaluation (25 of 25 mice for each of the three methods). *H. pylori* populations isolated from the gastric samples of mice in this group ranged from 3.3×10^4 to 2.6×10^6 CFU/g of gastric tissue (Fig. 2), with no statistical difference over the observation period. In these animals, *H. pylori* was detected on the mucosal surface and in the upper foveolae by histopathologic evaluation. At a higher magnification, the bacteria appeared to be adherent to the gastric epithelium (Fig. 3a). Colonization density was medium to high in the antrum (score, 2 or 3) but much lower in the body (score, 0 or 1) and the cardioesophageal junction. The mice developed chronic active gastritis (Fig. 3b) closely resembling the *H. pylori* gastritis observed in humans. At 1 month postinfection, the associated gastritis was evaluated for grade (presence of chronic inflammatory cells, scored 0 or 1) (Fig. 4a) and activity (neutrophilic infiltration, scored 0 or 2) (Fig. 5a), according to the updated Sydney system. However, in samples collected at 2 months postinfection, more severe gastritis was evident (chronic gastritis score, 1 or 2 [Fig. 4a], and active gastritis score, 1, 2, or 3 [Fig. 5a]). In animals with severe gastritis (grade score, 2; activity score, 3), the formation of lymphoid follicles also was observed (data not shown), as was that of intraluminal crypt neutrophilic abscesses (Fig. 3b). After 2 months postinfection, a gradual attenuation of active gastritis was observed (Fig. 5a), as reported before (20). In the body and cardioesophageal junction, the severity of gastritis was milder (grade score, 0; activity score, 1) (data not

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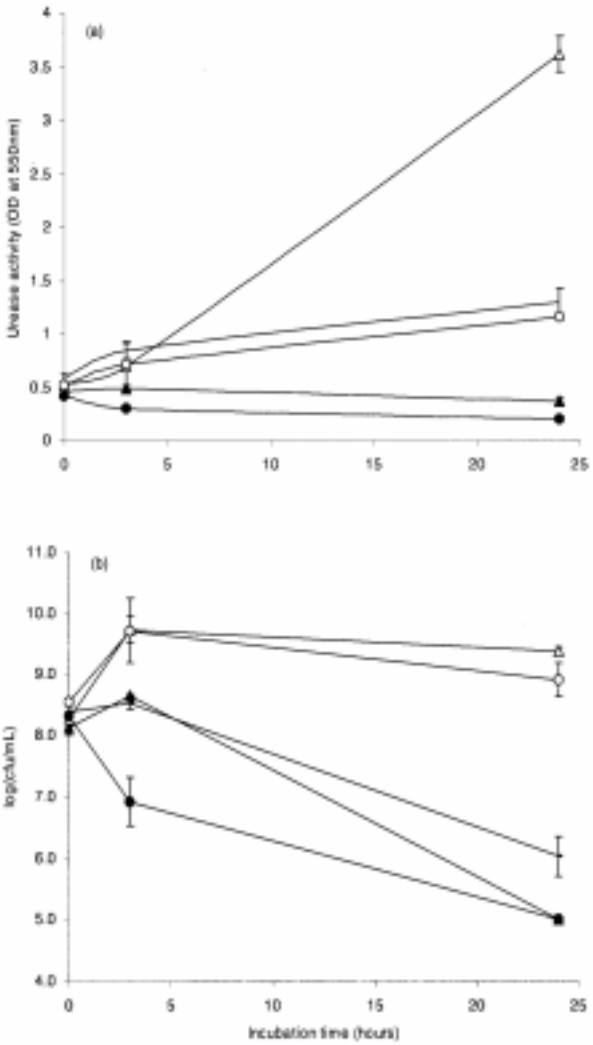

FIG. 1. In vitro effect of *L. casei* strain Shirota on *H. pylori* urease activity and viability. *H. pylori* SS1 cells (10^8 CFU/ml) suspended in BHIB without antibiotics were incubated under microaerophilic conditions at 37°C in the presence of a 10% volume of *L. casei* strain Shirota culture supernatant (pH 4.5 [●] or pH 6.5 [○]) or the appropriate MRS medium control (pH 4.5 [▲] or pH 6.5 [△]). Amoxicillin at 0.12 μg/ml (two times the MIC; solid line with no symbols) was included as a positive control for *H. pylori* growth inhibition. At 0, 3, and 24 h, samples were collected and assayed for urease activity and *H. pylori* viability. (a) Relative urease activity measured by introducing 10-μl *H. pylori* suspensions as described above to 300 μl of urease reaction buffer, incubating the mixtures for 2 h at 37°C, and measuring the optical density (OD) at 550 nm. Points represent the mean and standard error of the mean for three independent experiments. (b) *H. pylori* viability evaluated by determining viable counts on CHW agar plates following incubation at 37°C under microaerophilic conditions. Points represent the mean and standard error of the mean for three independent experiments. Note that there was a significant reduction in the urease activity of *H. pylori* cells incubated with *L. casei* strain Shirota supernatant at pH 6.5 (a), but the cells retained their viability (b). In the presence of 10% *L. casei* strain Shirota supernatant at pH 4.5 and the respective MRS medium at pH 4.5, control inhibition of urease activity and *H. pylori* viability was observed.

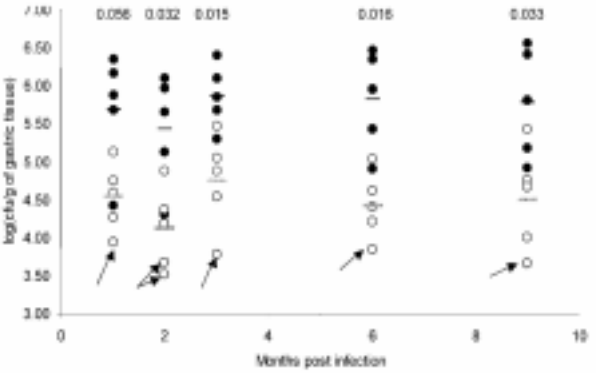

FIG. 2. *H. pylori* colonization of gastric mucosa in the *H. pylori* SS1 group (control group) (●) and the *H. pylori-L. casei* strain Shirota group (study group) (○). Each circle represents one animal. Lines represent the respective means in the control group (black lines) and the study group (gray lines). Numbers above each time point represent the *P* values calculated between the two groups at each time point (Mann-Whitney test; significant difference, $P < 0.05$). Arrows identify the six animals in the study group which were found *H. pylori* negative by histopathologic evaluation. Significant differences between the two groups, with respect to the number of colonizing bacteria, were observed at 2, 3, 6, and 9 months postinfection. No significant difference was observed within each group during the observation period.

shown). These results are consistent with the histopathologic scenario presented by the *H. pylori* SS1 mouse infection model (17).

For the *L. casei* strain Shirota group (control mice), given just the probiotic, gram-positive colonies isolated from the feces were initially classified by the API 50CHL test and verified by species-specific PCR. PCR was very effective in discriminating the administered *L. casei* strain Shirota from other lactobacilli, such as one *L. reuteri* and two *L. gasseri* strains also isolated in the normal gut flora of our mice. The administered *L. casei* strain Shirota was isolated at populations of 10^7 to 10^8 CFU/g of wet feces. However, it was not isolated or detected in washed intestinal tissue samples by species-specific PCR. In preliminary experiments, in which we examined the ability of *L. casei* strain Shirota to colonize the gut of healthy animals, we failed to continue isolating it in the feces approximately 20 days after the discontinuation of administration. All of the above results clearly indicate that *L. casei* strain Shirota does not colonize the gut epithelium and therefore will be present in the microflora only for nearly as long as it is administered. Histopathologic examination of gastric samples showed normal mucosa with the absence of gastritis in the antrum (Fig. 3c), body, and cardioesophageal junction.

We monitored weight as an index of the general well-being of the animals and found no significant difference among the groups of mice in the study. Although the presence of *H. pylori* in humans is related to type II gastritis, duodenal ulcer, mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma, there is no indication in the literature of an effect on the weights of *H. pylori*-infected persons or animals in the associated animal models. Finally, monitoring of water intake revealed that the animals consumed equal amounts of untreated water or *L. casei* strain Shirota-containing water. The daily probiotic dose was calculated to be 3×10^7 CFU/g of body weight.

Evaluation of in vivo *H. pylori* colonization and associated gastritis in *H. pylori*-infected animals treated with *L. casei* strain Shirota. For the *H. pylori-L. casei* strain Shirota group, *H. pylori* was detected in gastric samples by viable counts and PCR (25 of 25 mice for each of the two methods). Histopathologic evaluation revealed the presence of *H. pylori* in 19 of 25 mice. The difference in *H. pylori* populations (CFU per gram of gastric tissue) between the gastric samples of mice in the study group and those of mice in the control group was significant at 2 months postinfection and throughout the remainder of the 9-month observation period ($P < 0.05$; Mann-Whitney test) (Fig. 2). A significant decrease in chronic (Fig. 4b) and active (Fig. 5b) gastric mucosal inflammation also was observed at each time point throughout the observation period. The lamina propria usually contained a small amount of scattered lymphocytes (score, 0), as they are normally seen in the gastric mucosa despite the presence of *H. pylori* colonization (Fig. 3d). The grade of chronic gastritis was assessed as mild (score, 1) in 9 of 25 animals, because diffuse mild lymphocytic and plasmacytic inflammatory infiltration was observed (Fig. 4b). Mild neutrophilic infiltration of the lamina propria (activity score, 1) was observed in 11 of 25 animals (Fig. 5b). Significant differences in the body and the cardioesophageal mucosa with regard to *H. pylori* colonization and associated gastritis also were observed between the study and control groups (data not shown). *L. casei* strain Shirota was isolated from the intestinal contents throughout the entire administration period. However, albeit repeated attempts, isolation or detection of *L. casei* strain Shirota in washed intestinal tissue samples by PCR was not feasible.

Detection of anti-*H. pylori* IgG antibodies. All animals in the *H. pylori* SS1 group (control group) and the *H. pylori-L. casei* strain Shirota group (study group) were found to be positive for the presence of specific anti-*H. pylori* IgG antibodies in the serum samples collected, unlike the uninfected animals (Fig.

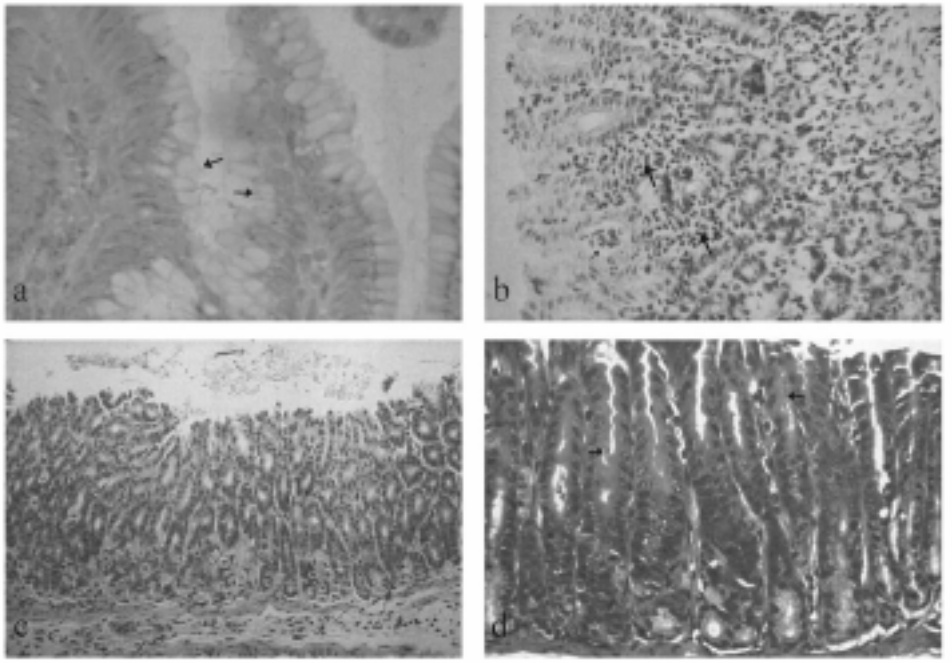

FIG. 3. Histopathologic evaluation of antral gastric samples from *H. pylori* SS1 group mice (a: Giemsa stain, magnification, ×316; b: H&E stain, magnification, ×158), *L. casei* strain Shirota group mice (c: H&E stain, magnification, ×79), and *H. pylori-L. casei* strain Shirota group mice (d: Giemsa stain, magnification, ×158). Note the presence of *H. pylori* in the gastric pits (a, arrows); the presence of chronic active inflammation in the lamina propria (b, large arrows), with the formation of intraluminal abscesses in the gastric pits (b, small arrow); the apparently normal mucosa in the *L. casei* strain Shirota group mice (c); and the absence of gastritis in the presence of *H. pylori* (d, arrows) in the *H. pylori-L. casei* strain Shirota group mice.

6). There were wide variations in the optical densities detected by the ELISA (ranging from 0.765 to 2.450) for serum samples at 6 and 9 months postinfection. A tendency for a reduction in antibody response in the *H. pylori-L. casei* strain Shirota study group was observed, although it was not statistically significant ($P > 0.05$; Mann-Whitney test).

DISCUSSION

In our study, inhibition against *H. pylori* SS1 and the nine wild-type clinical strains was evident with the agar diffusion assay only when there was a direct involvement of viable *L. casei* strain Shirota cells. No anti-*H. pylori* activity was obtained with a cell-free *L. casei* strain Shirota culture supernatant, whether the pH was preserved at 4.5 or adjusted to 6.5, possibly due to the buffering capacity of the medium and the horse blood serum proteins present. Our data are in complete accordance with recent observations in which *L. casei* strain Shirota culture supernatants isolated by plain centrifugation (containing as much as 10^4 CFU of viable bacteria/ml) were active in vitro against *H. pylori* in well diffusion assays; subsequent sterilization through 0.22-μm-pore-size filters abolished the inhibitory activity (5). Like us, the authors were unable to produce evidence of bacteriocin activity in the *L. casei* strain Shirota cell-free culture supernatants.

In our assays involving liquid *H. pylori* cultures, the addition of an *L. casei* strain Shirota culture supernatant (pH 4.5) and the respective MRS medium control (pH 4.5) decreased the final incubation pH to 5.1, resulting in the inhibition of urease activity and *H. pylori* viability. It is well documented that *H. pylori* viability in vitro under acid stress in the absence of urea is very low (30). Apart from the resulting low pH, others utilizing in vitro inhibition screening methods have attributed such anti-*H. pylori* activity of lactobacilli to lactic acid production (1, 2). More specifically, in cocultures of *H. pylori* and *L. salivarius*, concentrations of more than 10 mM lactic acid produced by the lactobacilli in the incubation medium strongly inhibited urease activity and *H. pylori* viability (1). When we incubated *H. pylori* with lactic acid concentrations of 100 to 1 mM, we observed over 70% inhibition of urease activity for 15 mM lactic acid within 3 h of incubation. In our liquid culture assays in which only 10% *L. casei* strain Shirota culture supernatant was present, the lactic acid concentration measured by HPLC was in about 15 mM, a concentration capable of induc-

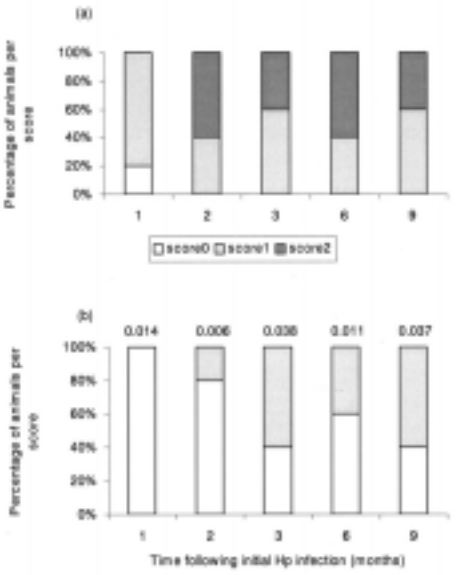

FIG. 4. *H. pylori*-associated chronic gastritis in the antrum. Bars represent the percentages of samples with each histopathologic score (0, 1, or 2) in the *H. pylori* (Hp) SS1 group (control group; five animals per time point) (a) and the *H. pylori-L. casei* strain Shirota group (study group; five animals per time point) (b). Numbers above the columns in panel b represent the *P* values calculated between the two groups at each time point, with reference to *H. pylori* chronic gastritis score. Significant differences were observed at all time points during the observation period (Wilcoxon rank sum test).

ing the inhibition of *H. pylori* urease activity and viability. Furthermore, when an *L. casei* strain Shirota culture supernatant adjusted to pH 6.5 was used, there was significant inhibition of *H. pylori* urease activity, although no reduction in viable counts was observed over 24 h, possibly due to the absence of acid stress on *H. pylori*. Collectively these results suggest that lactic acid produced by *L. casei* strain Shirota is involved in inhibition of the bacterial urease system. This activity of lactic acid is probably a combination of its inhibitory effect on the bacterial urease system (1) and the reduced ability of *H. pylori* to survive at a low pH in the absence of urea (30). In our experimental design, urease inhibition by lactic acid contained in the *L. casei* strain Shirota culture supernatant can render *H. pylori* incapable of overcoming the low-pH conditions. In the respective MRS medium control (pH 4.5) acidified with HCl, in which the urease system is functioning, small amounts of endogenous urea (23) could sustain viability for the first few hours, but not over the entire 24-h incubation period.

The exact mechanism by which lactic acid inhibits urease activity remains elusive. However, others (5, 7) have observed very little or no effect on urease activity attributed to lactic acid, although direct comparisons with our work are impossible due to the different experimental designs used for the determination of lactic acid. We have studied in detail by HPLC the kinetics of lactic acid production in *L. casei* strain Shirota cultures grown in MRS medium and found lactic acid to be the main product (at concentrations of up to 150 mM). Furthermore, only traces of lipid are present, attributed to the 0.1% Tween detergent in the MRS medium, and *L. casei* strains in general have low proteolytic activity compared to other lactobacilli, such as *L. bulgaricus*. Finally, competition for nutrients can be ruled out, as we did not observe inhibition of *H. pylori* when *E. coli* was used as a positive control for nutrient depletion in cultures.

Since its introduction, the *H. pylori* SS1 mouse infection model has been used extensively in the field of *H. pylori* research, and the conditions for its application have been described in detail (12, 17). We used the particular infection model in which animals were treated with large doses of *L. casei* strain Shirota in a continuous fashion through the water supply. *L. casei* strain Shirota exhibited good viability in water; therefore, we succeeded in giving it to the animals through the normal water supply, in effect alleviating the burden of repeated orogastric administrations (35). Furthermore, this method of administration was superior to mixing freeze-dried

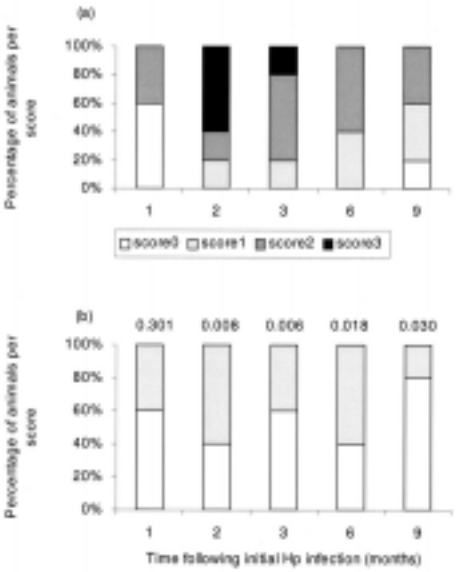

FIG. 5. *H. pylori*-associated active gastritis in the antrum. Bars represent the percentages of samples with each histopathologic score (0, 1, 2, or 3) in the *H. pylori* (Hp) SS1 group (control group; five animals per time point) (a) and the *H. pylori-L. casei* strain Shirota group (study group; five animals per time point) (b). Numbers above the columns in panel b represent the *P* values calculated between the two groups at each time point, with reference to *H. pylori* active gastritis score. Significant differences in gastritis activity were observed after month 2 and throughout the entire observation period thereafter (Wilcoxon rank sum test). After month 2, a gradual attenuation of the phenomenon within the *H. pylori* SS1 group was observed, unlike the results for the *H. pylori-L. casei* strain Shirota group.

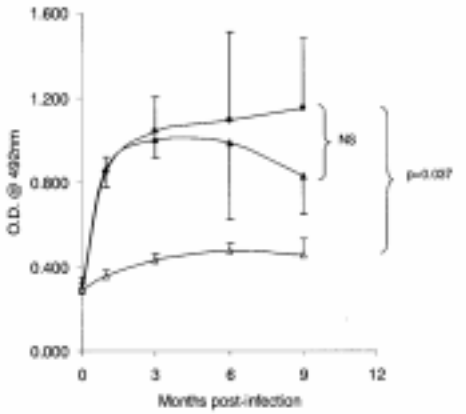

FIG. 6. Total IgG response to *H. pylori* infection in C57BL/6 mice. Serum samples collected at 0, 1, 2, 3, 6, and 9 months postinfection were assayed for anti-IgG *H. pylori* antibodies by an ELISA. Although there was a decrease in the IgG response in the *H. pylori-L. casei* strain Shirota group (study group; five animals per time point; ▲) compared to the results in the *H. pylori* SS1 group (control group; five animals per time point; ●), this decrease was not found to be significant (Mann-Whitney test). Open triangles represent the *L. casei* strain Shirota group (control group; five animals per time point). O.D., optical density; NS, not significant.

Lactobacillus cultures in animal feed, because we could monitor the viability as well as the concentration of the bacteria administered. Finally, it provided a continuous administration of lactobacilli, thus maintaining a constant "therapeutic dose" to the animals. We observed no differences in the drinking habits of the animals due to the presence of lactobacilli, as consumption of water containing lactobacilli was no different from consumption by the control animals, which received only plain water.

Monitoring the kinetics of *L. casei* strain Shirota after ingestion is a formidable task, because of the plethora of microbes present in the normal mouse intestinal microflora. Numerous existing methods encompass classical culture-dependent techniques, with selective or nonselective media, and culture-independent techniques, including microscopy, enzyme metabolite analysis, and molecular sequence analysis (26). We were able to isolate *L. casei* strain Shirota from intestinal contents by using selective media as well as to discriminate it by species-specific PCR from other lactobacilli contained in the normal flora of the mouse. For the *H. pylori-L. casei* strain Shirota group, we observed a significant reduction of *H. pylori* colonization in the gastric mucosa throughout the entire observation period (9 months). In our experiments, *H. pylori* colonization in the range of 10^4 CFU/g was hardly detectable by histopathologic evaluation, an observation made by others as well (31). We therefore based our conclusion regarding *H. pylori* colonization on data obtained by quantitative isolation of the bacterium from dissected gastric samples rather than just by histopathologic evaluation. Such a protocol has been used routinely for the assessment of *H. pylori* viable counts in the SS1 mouse model (12, 17) and has proved very efficient in measuring the colonization efficiency of *H. pylori* colonization-deficient mutant strains with gene knockouts (23). Also, to the best of our knowledge, there is no literature supporting the formation of biofilms by *H. pylori*. Whether this in vivo suppressive effect of *L. casei* strain Shirota on *H. pylori* is associated with the observed in vitro inhibition of bacterial urease activity, which is essential for the survival of *H. pylori* in the stomach, remains to be further documented. In a similar study, *L. salivarius* was able to suppress *H. pylori* in vivo in an *H. pylori*-infected gnotobiotic murine model (1). The authors were able to measure lactic acid concentrations of between 2.3 and 18.9 μmol/g in the gastric contents and found that such concentrations inhibited *H. pylori* viability and completely abolished urease activity in vitro. In a recent intervention study in which the effect on *H. pylori* colonization was assessed by the UBT, *L. casei* strain Shirota-containing milk administered to *H. pylori*-colonized subjects had a slight but nonsignificant suppressive effect on *H. pylori* (5). In view of the data on urease inhibition by lactic acid, methods more stringent than UBT (i.e., determination of viable counts or histopathologic evaluation) should be used for the evaluation of *H. pylori* colonization in clinical studies involving lactic acid bacteria with a potential for inhibition of urease activity. In our study, the observed significant attenuation in the chronic and acute inflammation of the gastric mucosa, with no to moderate lymphoplasmacytic infiltration in the lamina propria, can be attributed primarily to the reduced *H. pylori* colonization levels in the *L. casei* strain Shirota-treated mice. However, involvement of a systemic mechanism with a possible immunological background cannot be ruled out. Indeed, studies on the immunological effects of orally administered *L. casei* strain Shirota have highlighted its ability to modulate immune responses in the establishment of tumor immunity and the induction of specific antitumor activity (21, 22). In addition, *L. casei* strain Shirota promoted the production of several different cytokines involved in the regulation of host cellular immune responses, resulting in the alteration of lymphocyte susceptibility to apoptosis (16). Finally, *L. casei* strain Shirota was able to increase cellular immunity significantly, as determined by the delayed-type hypersensitivity response to heat-killed *L. monocytogenes*, and therefore to enhance the host response against oral *L. monocytogenes* infection in rats (8). Therefore, the stimulation of specific immunity and nonspecific immunity (16, 18, 25, 28) could be another mechanism by which this particular probiotic exerts its anti-*H. pylori* activity, a hypothesis currently being investigated in our laboratory. A reduction of *H. pylori*-associated gastritis also was reflected by the marked yet not significant reduction in the anti-*H. pylori* IgG response detected in the study group animals over the 9-month observation period. Aiba et al. (1) observed a significant reduction of antibody titers to *H. pylori* in mice dosed with *L. salivarius*, but they did not evaluate *H. pylori*-associated gastritis by histopathologic analysis.

However complex the inhibition mechanism may be, the fact remains that significant suppression of the colonization ability of *H. pylori* and the associated inflammation was evident in *L. casei* strain Shirota-treated animals. To our knowledge, this is the first documented report of *L. casei* strain Shirota administration promoting a decrease in *H. pylori* colonization and a significant attenuation of the associated gastritis in a well-

established animal model of *H. pylori* infection. We believe that our results also could be related to the continuous mode of administration of the probiotic through the water supply, in combination with the high doses given to the animals. We have succeeded in administering doses of lyophilized *L. casei* strain Shirota of higher than 50 mg, a daily dose of approximately 5×10^9 CFU. This daily dose expressed in the human condition amounts to as much as 175 to 200 g of lyophilized *Lactobacillus* culture, assuming an average human weight of 70 kg. It is clear that there is a lack of data on an effective therapeutic dose for probiotics. In comparison to doses used in existing clinical trials evaluating the efficacy of probiotics, this dose is potentially a very high dose sustained over a period of 9 months. It is conceivable that the probiotic effect of lactic acid bacteria would be best manifested if the bacteria were administered at high doses in a continuous fashion; however, such an approach needs to be proven in an appropriate clinical setting.

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This study does not necessarily reflect the views of the Commission of the European Communities and in no way anticipates the Commission's future policy in this area.

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ORIGINAL ARTICLE

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healthsciences

Clinical Significance of Cytotoxin-associated Gene A Status of *Helicobacter pylori* Among Non-steroidal Anti-inflammatory Drug Users with Peptic Ulcer Bleeding: A Multicenter Case-Control Study

D. Tzourmakliotis, M. Economou, S. Manolakopoulos, S. Betharis, C. Bergele, J. Lakoumentas, P. Sclavos, H. Milionis, A. Margeli, E. Vogiatzakis & A. Avgerinos

Dept. of Gastroenterology and the Depts. of Cardiology and Microbiology, Polyclinic General Hospital, Vrilissia, Athens, Greece; 2nd Dept. of Gastroenterology Evangelismos General Hospital, Athens, Greece

Tzourmakliotis D, Economou M, Manolakopoulos S, Betharis S, Bergele C, Lakoumentas J, Sclavos P, Milionis H, Margeli A, Vogiatzakis E, Avgerinos A. Clinical significance of cytotoxin-associated gene A status of *Helicobacter pylori* among non-steroidal anti-inflammatory drug users with peptic ulcer bleeding: a multicentre case-control study. Scand J Gastroenterol 2004;39:1180–1185.

Background: The role of *Helicobacter pylori* infection and especially of the cytotoxin-associated gene A (CagA) product strain in peptic ulcer bleeding among non-steroidal anti-inflammatory drugs (NSAIDs) users remains controversial. **Methods:** A case-control study was carried out including 191 consecutive chronic NSAIDs users admitted to hospital because of peptic ulcer bleeding. Peptic ulcer was verified by endoscopy. Controls comprised 196 chronic NSAIDs users without signs of bleeding of similar age and gender to cases. Multivariate regression analysis was performed for farther evaluation of the relationship between *H. pylori*, CagA status and other risk factors. **Results:** *H. pylori* infection was present in 121 (63.4%) cases compared with 119 (60.7%) controls (odds ratio (OR) = 1.14, 95% CI, 0.76–1.72). CagA-positive strains were found to be significantly more frequent in cases than in controls (65/106 versus 41/99 $P = 0.008$). Current smoking (OR = 2.65; 95% CI, 1.14–6.15; $P = 0.02$), CagA status (OR = 2.28; 95% CI, 1.24–4.19; $P = 0.008$), dyspepsia (OR = 6.89; 95% CI, 1.84–25.76; $P = 0.004$) and past history of peptic ulcer disease (OR = 3.15; 95% CI, 1.43–6.92; $P = 0.004$) were associated significantly with increased risk of bleeding peptic ulcer. **Conclusions:** The results suggest that CagA-positive *H. pylori* infection is associated with a more than 2-fold increased risk of bleeding peptic ulcer among chronic NSAIDs users.

Key words: Bleeding; CagA; *H. pylori*; NSAIDs; peptic ulcer

Dimitrios Tzourmakliotis, 40B Marathonos Street, GR-15235 Vrilissia, Greece (fax. +30 210 5234 108, e-mail. ahc04035@otenet.gr)

Helicobacter pylori infection and non-steroidal anti-inflammatory drugs (NSAIDs) are the two most important independent risk factors for peptic ulcer disease and its serious complications (1, 2). However, whether the underlying *H. pylori* infection increases the risk of peptic ulcer complications in NSAIDs users is still a matter of debate with some studies showing an increased risk of peptic ulcer bleeding (3–6) and others concluding the opposite (7–9). Furthermore, conflicting results have been reported on whether *H. pylori* eradication reduces the risk of peptic ulcer disease and its complications in NSAIDs users (10–15). However, the exceptionally widespread use of NSAIDs worldwide makes a deeper investigation of all the parameters concerned in this important health-care issue necessary.

There is evidence that strains of *H. pylori* differ in their association with gastrointestinal diseases and there is genetic diversity in the *H. pylori* species population (16). It has been suggested that infection with the *H. pylori* strain expressing the cytotoxin-associated gene A product (CagA positive) is more closely associated with peptic ulcer disease (17, 18). Whether *H. pylori* infection with these virulence factors increases the risk of peptic ulcer bleeding in patients taking NSAIDs is still under investigation. Moreover, less is known about other risk factors and how they relate to NSAIDs and *H. pylori* strains expressing this virulence factor.

The aims of this study were: 1) to determine whether *H. pylori* infection is a risk co-factor for peptic ulcer bleeding in chronic NSAIDs users and 2) to determine whether *H. pylori* expressing the CagA virulence factor and other potential factors affect the risk of bleeding in this population.

Patients and Methods

Patients with signs of upper gastrointestinal bleeding (haematemesis, melena) admitted to our units from December

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1999 to May 2002 were eligible for the study if they met the following criteria: age >18 years, chronic NSAIDs use including aspirin in analgesic or antithrombotic doses and peptic ulcer verified by endoscopy performed within 18 h after admission. Peptic ulcer was defined as a circumscribed mucosal break ≥5 mm with well-defined crater. Chronic use was defined as NSAIDs intake at least three times per week for at least 6 months; at least one dose had to have been taken in the week before the gastrointestinal bleeding episode (12). We excluded patients with upper gastrointestinal cancer, a history of gastric-intestinal surgery, evidence of perforation, known cirrhosis and portal hypertension, terminal-stage illness in which endoscopy was contraindicated and patients that were unable to give a history. Of the 311 patients admitted to our units with signs of upper gastrointestinal bleeding during the study period, 120 were excluded because of ineligibility according to the inclusion and exclusion criteria. Data are not available for screened patients who did not meet the inclusion criteria.

Eligible controls were chronic NSAIDs, including aspirin, users without signs of ulcer bleeding at the time of the interview (19, 20) recruited from hospitalized patients in the Cardiology and Gastroenterology Depts. Because the risk of bleeding peptic ulcer depends on age and gender (21), controls were frequency-matched by age (±10 years) and by gender according to established practice (22). The controls were eligible if their primary diagnosis was neither an indication for nor a known complication of treatment with NSAIDs.

Cases and controls were interviewed by research associates who were aware of the specific study hypotheses. Structured questionnaires covered potential risk factors such as history of previous upper gastrointestinal diseases including a history of peptic ulcer disease, presence of dyspeptic symptoms, current smoking habits, alcohol consumption, use of oral corticosteroids, anticoagulants and concomitant diseases. Dyspepsia was defined as chronic or recurrent pain or discomfort centred in the upper abdomen within the previous three months (23). Alcohol consumption of more than 50 mg/day was included as a potential risk factor in the study. Aspirin and non-aspirin NSAIDs use was recorded separately.

All patients and controls gave their full informed consent and the study was approved by the local ethics committee. The research was carried out in accordance with the Helsinki Declaration, as revised in 1989.

H. pylori infection

Blood from patients and controls was sampled at admission for serologic analysis and all sera were aliquoted and stored frozen at −80 °C until serological studies were performed.

In patients, the presence of *H. pylori* infection was determined by a rapid urease test (Campylobacter-like organisms test (CLO test) Delta West Ltd., Bentley, Australia) from biopsies taken at the antrum and by identification serum IgG antibodies to *H. pylori* in duplicate by a second-generation enzyme-linked immunosorbent commercial assay with high sensitivity (90%) and specificity (97%) (Anti-*H. pylori* EIA Quant, Roche Diagnostics GmbH, Germany). According to the manufacturer, an immunoglobulin (Ig) G level of >6.6 mL was considered positive. In cases with indeterminate values (5.4–6.6 U/mL) a second evaluation was performed. Numerous publications have shown that this and other serological tests distinguish between those who are currently infected with *H. pylori* and those who are not (24, 25).

A patient was considered *H. pylori*-positive if both the rapid urease test and the serological test were positive. In controls, the serum IgG antibodies to *H. pylori* were used for *H. pylori* determination

CagA

The anti-CagA assay was done using commercial ELISA kits (Genesis Diagnostics Ltd., UK) according to the manufacturer's instructions by one single person blinded to cases and controls. Briefly, purified recombinant CagA protein is coated onto the surface of the wells. Serum specimens (diluted 1:200) are incubated for 30 min to allow antibodies to CagA protein to bind to the antigen in the wells. After washing away unbound antibodies and other serum constitutes, CagA-specific IgG is detected using rabbit anti-human IgG conjugated to horseradish peroxidase. After 30 min of incubation, the unbound conjugate is removed by washing and TMB enzyme substrate is added. Optical density was obtained at 405 nm using a microplate reader.

The ELISA technique has previously been validated and has a sensitivity of 96% and specificity of 97% for the detection of carriage of CagA-positive strains. (26) The cut-off values for the ELISA as recommended by the manufacture were: <5 units negative; 5.5–7 units, borderline; >7 units positive. Control sera provided with the kit were used for semiquantitative analysis.

Statistical analysis

Age data are summarized by the mean and standard error of the mean presented as the "mean ± s_E (standard error of the mean)". In order to investigate the prevalence of bleeding with respect to the various risk factors recorded in this study, logistic regression was used to quantify the influence of these factors. The backward stepwise (Likelihood Ratio) multivariate logistic regression analysis performed using the statistical software program SPSS 10.0 (SPSS Inc., Chicago, Ill., USA) included the potential risk factors of gender, age, history of peptic ulcer, dyspepsia within 3 months, *H. pylori* and CagA status, NSAIDs (including aspirin) usage, smoking habits and alcohol consumption. In all cases, a P value <0.05 was taken as significant and odds ratios (ORs) 95% confidence intervals (CI) were calculated. Univariate analysis was performed with the Fisher exact test or chi-squared test with Yates's correction where appropriate.

It is recognized that there was multiple testing of outcome data arising from individual patients. The P values presented

are without correction, but it is indicated wherever application of the Bonferroni method would remove statistical significance from any findings from the definitive multivariate logistic regression analyses. The *P* values from the univariate analyses (comparing groups) should be taken as explanatory only, to highlight variables to be included in the definitive multivariate models.

Results

Characteristics of patients and controls

A total of 387 ($n = 387$) consecutive patients were included in the study. A total of 191 patients (118 M (61.8%)) with a mean age of 66.4 ± 12.9 years (range, 23–87) had peptic ulcer bleeding; 10 (5.2%) were younger than 40 years of age, 164 (85.8%) were between 40 and 80 years of age and 17 (8.9%) were older than 80 years of age. The control population consisted of 196 patients (113 M (57.7%)) with a mean age of 67.9 ± 10.0 years (range, 43–92); 176 of these subjects (89.8%) were between 40 and 80 years of age, and 20 (10.2%) were older than 80 years.

Prior to admission, 120 patients (62.8%) had taken NSAIDs compared to 28 (14.3%) of the controls, and 71 (37.2%) patients had taken aspirin compared with 168 (85.7%) of the controls ($P < 0.0001$). Most of the patients and controls had been using aspirin for cardiovascular prophylaxis and the most common reason for using non-aspirin NSAIDs was musculoskeletal disorders. Co-use of aspirin and NSAIDs was not seen.

There were more current smokers (23%) and patients with a history of peptic ulcer (37%) in the group of case subjects than controls (8.2% and 11.2%, respectively, $P = 0.0001$, $P < 0.0001$). No difference between the two groups was observed in alcohol consumption. Anticoagulant therapy was used in only 1 patient and in 2 controls. Analysis of this variable was precluded.

Reasons for admission in case subjects were haematemesis in 24 (13.3%) patients and melena in 157 (86.7%). Thirty-six cases (18.4%) presented signs of hypovolaemia during admission. Endoscopy revealed upper gastrointestinal bleeding caused by duodenal ulcer in 112 (58.7%) of the patients, gastric ulcer in 63 (33.0%) and a combination of gastric and duodenal ulcer in 16 (8.3%) patients. Twenty-eight patients had evidence of blood in the stomach at endoscopy; three of them had negative CLO tests with serological evidence of *H. pylori* infection and the diagnosis of *H. pylori* infection was established only by serology.

Neither the controls nor the patients were treated with *H. pylori* eradication therapy during the past 12 months and had systematically received proton-pump inhibitors or H_2-receptor blockers at least one week before entering the study. Fifteen patients and 21 controls reported use of antibiotics before admission. Only 14 cases had concomitant diseases; we therefore excluded this small group from the multivariate analysis.

H. pylori and CagA status—determinants of risk of ulcer bleeding

Information on *H. pylori* infection was available in all cases and controls. *H. pylori* infection was present in 121 (63.4%) patients compared with 119 (60.7%) controls. Among the 121 patients with evidence of *H. pylori* infection, 77 (63.7%) were diagnosed with duodenal ulcer, 39 (32.2%) with gastric ulcer and 5 (4.1%) patients had both duodenal and gastric ulcers. The crude OR for bleeding peptic ulcer associated with *H. pylori* infection among NSAIDs users was 1.14 (95% CI, 0.75–1.72).

Sera samples of 205 *H. pylori*-positive patients (106/121) and controls (99/119) were available for determination of antibodies against CagA *H. pylori* antigen. The above subgroups of cases and controls were similar with regard to age, gender, smoking, alcohol intake and history of peptic ulcer. CagA-positive strains were found to be significantly more frequent in patients than in controls (65 (61%) versus 41 (41%), $P = 0.008$). Forty patients with CagA-positive strains were diagnosed with duodenal ulcer, 22 had gastric ulcer and 3 patients had both duodenal and gastric ulcers. The crude ORs for bleeding peptic ulcer among NSAIDs users are presented in Table I.

Using our data on admission, we assessed the potential risk factors for bleeding peptic ulcer among chronic NSAIDs users. We studied 8 variables: gender, age, current smoking, dyspepsia, alcohol consumption, past history of peptic ulcer disease, H. pylori infection and CagA status. In the multivariate logistic regression model, where the effect of each factor is adjusted for the effect of each of the others in the model, smoking, dyspepsia, CagA status and history of peptic ulcer were significantly associated with an increased OR for bleeding peptic ulcer (Table II).

We performed a further multivariate analysis by dividing our patients according to *H. pylori* status and by investigating two multivariate models. We observed that a past history of peptic ulcer remained significantly associated with an increased OR for bleeding peptic ulcer, even when there was no *H. pylori* infection (Table III).

In order to investigate whether the effect of *H. pylori* infection and CagA status were confined to any specific site of bleeding, we performed a subgroup analysis according to the gastric and duodenal ulcers (Table IV). Dyspepsia, a history

Table I. Crude odds ratios of bleeding peptic ulcer among NSAIDs users associated with potential risk factors

	Odds ratio (95% CI)	P^1
Current smoking	3.37 (1.83–6.21)	0.0001
Dyspepsia	1.99 (1.02–3.86)	0.04
Past history of peptic ulcer	4.68 (2.79–7.96)	0.0001
CagA	2.24 (1.28–3.92)	0.005

CaGA = cytotoxin-associated gene A; NSAIDs = non-steroidal anti-inflammatory drugs.
[1]These *P* values are uncorrected for multiple testing since these comparisons are explanatory only, to highlight variables to be included in the definitive multivariate models.

Table II. Adjusted odds ratios of bleeding peptic ulcer among NSAIDs users associated with potential risk factors

	Odds ratio (95% CI)	*P*
Current smoking	2.65 (1.14–6.15)	0.023[1]
Dyspepsia	6.89 (1.84–25.76)	0.004
Past history of peptic ulcer	3.16 (1.44–6.92)	0.004
CagA	2.28 (1.25–4.19)	0.008

CaGA = cytotoxin-associated gene A; NSAIDs = non-steroidal anti-inflammatory drugs.
[1]The Bonferroni correction of the *P* value for 'Current Smoking' removes statistical significance.

of peptic ulcer and CagA positivity were found to be significant independent predictors of bleeding, independently of the site of ulcer. It is of interest that male gender is associated with a 2-fold doubled OR for bleeding due to duodenal ulcer among chronic NSAIDs users.

A single multivariate model can be formulated based on Table II. In this model the OR for peptic ulcer bleeding versus baseline = $e^{0.97*\text{smoking} + 1.93*\text{dyspepsia} + 1.15*\text{past history} + 0.83*\text{CagA}} = e^{(0.97*\text{smoking})}e^{(1.93*\text{dyspepsia})}e^{(1.15*\text{pasthistory})}e^{(0.83*\text{CagA})}$, where a risk factor takes the value +1, if it is present, and 0, if not present. Then it is noted that, for example, if dyspepsia is present $e^{1.93*\text{dyspepsia}} = e^{1.93} = 6.89$, and if dyspepsia is absent, $e^{1.93*\text{dyspepsia}} = e^{0} = 1$, etc.; see Table II for the values of the other terms (individual ORs) when the factor is present. The overall OR for a given set of factors, then, is the product of the individual ORs for the factors present. Thus, a current smoker who is taking NSAIDs has 2.65-fold increased odds of bleeding peptic ulcer than a non-smoker, while a current smoker with a past history of peptic ulcer, no dyspepsia and who is CagA negative has a 2.65 * 3.16 = 8.36 times greater odds of bleeding compared to baseline.

Discussion

The primary aim of this case-control study was to examine whether *H. pylori* infection and particular CagA-positive strains are risk factors for bleeding peptic ulcer among chronic NSAIDs users. We have studied a sizeable cohort of consecutively selected patients admitted with signs of upper gastrointestinal bleeding and a similar number of age- and gender-matched controls; bleeding peptic ulcer was verified in all cases by endoscopy.

The results of our study have clearly shown an association between bleeding peptic ulcer and *H. pylori* infection that was significant only when there was serological evidence of infection with the CagA-positive *H. pylori* strain. In addition, our results further confirm that a past history of ulcer, smoking and dyspepsia appear to be independent risk factors for upper gastrointestinal bleeding among chronic NSAIDs users; the significance for smoking was removed by Bonferroni correction, indicating a weaker association with bleeding.

Although a number of studies have been performed to assess the relationship between *H. pylori* infection and NSAIDs use, controversy still exists. Four studies including the present study have shown no association between the two factors (7, 27, 28); one study has shown an increased risk of bleeding with *H. pylori* infection (3), while the findings of three studies suggest that the risk may be reduced in some circumstances (6, 8, 29). The reason for this discrepancy is unclear. Perhaps the different methodologies between studies in association with a complex relation between *H. pylori* infection and NSAIDs damage may explain the conflicting results. In a recent meta-analysis, Huang and colleagues (30) reported that *H. pylori* infection increases the risk of peptic

Table III. Adjusted odds ratios of bleeding peptic ulcer among NSAIDs users associated with potential risk factors according to *H. pylori* status

	H. pylori positive		*H. pylori* negative	
	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*
Current smoking	2.62 (1.13–6.08)	0.025[1]	4.54 (1.53–13.46)	0.006
Dyspepsia	6.10 (1.61–23.01)	0.008	–	
Past history	3.15 (1.44–6.93)	0.004	4.54 (1.88–10.99)	0.001
Alcohol	–		0.34 (0.10–1.11)	0.07
CagA	2.35 (1.28–4.32)	0.006	–	

CaGA = cytotoxin-associated gene A; NSAIDs = non-steroidal anti-inflammatory drugs.
[1]The Bonferroni correction of the *P* value for 'Current Smoking' in the *H. pylori*-positive subgroup removes statistical significance.

Table IV. Adjusted odds ratios of bleeding peptic ulcer among NSAIDs users associated with potential risk factors according to the site of peptic ulcer

	Duodenal ulcer		Gastric ulcer	
	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*
Dyspepsia	7.33 (1.82–29.44)	0.005	8.29 (1.87–36.77)	0.005
Peptic ulcer history	3.68 (1.5–9.04)	0.004	3.32 (1.27–8.69)	0.01
CagA	2.09 (1.05–4.16)	0.03[1]	2.76 (1.20–6.33)	0.01

CaGA = cytotoxin-associated gene A; NSAIDs = non-steroidal anti-inflammatory drugs.
[1]The Bonferroni correction of the *P* value for 'CagA' in the duodenal ulcer subgroup removes statistical significance.

ulcer bleeding in patients using NSAIDs. However, a large study that was not included in this meta-analysis showed an opposite effect (29); another clinical trial in which ulcer complications were the primary end-point did not support a role for *H. pylori* (31). The validity of the meta-analysis is therefore questionable owing to significant heterogeneity.

Endoscopic studies have shown that the CagA-positive strains have been associated with more severe forms of gastroduodenal damage (32, 33). The role of *H. pylori* expressing the virulence marker CagA in peptic ulcer bleeding is not fully understood. Our results are compatible with those reported by Stack et al. (29), who observed a higher risk of bleeding in cases with CagA-positive H. pylori infection. On the other hand, Santolaria et al. (6) and Lanas et al. (34) did not observe a significant increase in risk of bleeding in cases with a NSAIDs and CagA-positive relationship. The different design of the studies, the small number of patients included and the variability of the patient selection may be the reasons for this discrepancy.

We recruited only NSAIDs users bleeders because our primary aim was to investigate the role of *H. pylori* infection among NSAIDs users. Therefore, a straightforward estimation of the interaction between NSAIDs use and H. pylori infection as risk factors for bleeding peptic ulcer could not be achieved. It may be argued that in the present study the control collection may lead to possible sources of bias. A weak point of our study is the different diagnostic criteria used for establishing *H. pylori* infection between two groups (bleeders versus controls); however, we believed that performing an endoscopy in asymptomatic controls is unethical and, in addition, not a cost-effective strategy. Nevertheless, the 60% prevalence of *H. pylori* infection among controls is similar to the *H. pylori* prevalence in the country population of comparable age (35). Furthermore, hospitalized patients usually give more accurate histories than community controls, and previous studies have shown that hospital and community controls have similar rates in non-aspirin, NSAIDs use (36). Another possible limitation of the study concerning the heterogeneity of NSAIDs must be excluded, as no NSAIDs including prophylactic aspirin have yet proven to be without gastrointestinal toxicity (37, 38).

Diagnostic biases regarding the *H. pylori* (including CagA) status were unlikely because the diagnostic procedure was performed blinded to cases or controls. We used serotyping because this is highly sensitive and specific for current infection and does not have the limitation of the endoscopy-based CLO method in the presence of gastrointestinal bleeding (39). While *H. pylori* antibodies take up to a year to decline to indistinguishable levels after successful eradication (40), none of the patients and controls in our study had previous specific *H. pylori* eradication. In this kind of population, serology is a reliable index of current infection (41).

As our study was designed to suit the primary-care setting, we examined other risk factors extracted from patient questionnaires. Like others, we found that a history of peptic ulcer was an independent risk factor for bleeding among NSAIDs users (3, 4, 27), indicating that individual susceptibility is also responsible for the complication of bleeding in multifactorial peptic ulcer disease. We also estimated that smoking at least doubled the odds of peptic ulcer bleeding (with significance removed by Bonferroni correction for multiple testing), but alcohol intake was not related, probably owing to different population habits and the age of the study population. Our finding that dyspepsia is associated with bleeding peptic ulcer in NSAIDs users is in accordance with other investigators. Hansen et al. (42) reported that dyspepsia had a borderline significant 2-fold increase in risk of ulcer complications in patients taking NSAIDs. Although dyspepsia is not generally associated with endoscopic lesions and it is common in NSAIDs users (43, 44), our findings provide support for a role of dyspepsia in peptic ulcer bleeding in *H. pylori*-positive NSAIDs users. The difficulties in the definition of dyspepsia in association with the different design of the studies may explain the above conflicting results.

In summary, CagA-positive *H. pylori* infection, dyspepsia, history of peptic ulcer and current smoking are independent risk factors for bleeding peptic ulcer in chronic NSAIDs users. In the forthcoming era of the progressively increasing use of aspirin and other NSAIDs, further investigation with prospective well-designed studies are necessary in order to identify the high risk for bleeding NSAID users and the recommended strategies for *H. pylori* eradication.

Until then, according to our data, *H. pylori* eradication in CagA-positive subjects is necessary among NSAIDs users; prophylactic administration of anti-ulcer drugs seems a reasonable approach for all high-risk patients.

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Correspondence

Helicobacter pylori infection and the risk for coronary artery disease

To the Editor

We have read with interest the paper by Prof. Al-Nozha and colleagues, who found no association of coronary artery disease (CAD) with *Helicobacter pylori* (*H. pylori*) infection.[1] The authors advocated that "our patients, presented in this study, being of younger age group strengthens our negative finding of a causal relation of *H. pylori* as an independent risk factor for CAD". Indeed, they included patients as young as 16 years of age (mean 37.8 years), which makes it very unlikely for these patients to have an established CAD. Atherosclerosis is a gradual process being more frequent at older ages, and is associated with various factors. For instance, fibrinogen is a plasma protein that is converted into fibrin in the common pathway of coagulation. It remains significantly associated with the severity of coronary atherosclerosis in a progressive manner, even when adjustment is made for the age, hypertension, dyslipidemia, cigarette smoking, and body mass index.[2] Accumulating data shows that the increase in fibrinogen level during *H. pylori* infection and the high incidence of *H. pylori* seropositivity in patients with CAD represent a link between chronic *H. pylori* infection and CAD.[3,4] However, few studies show a lack of association between *H. pylori* infection and angiographically documented CAD. The observed discrepancy could be explained, at least in part, by the differences in various parameters of the population. Our study demonstrated that increased fibrinogen levels (an independent risk factor for CAD[2]) are associated with *H. pylori* infection and can be significantly reduced by *H. pylori* eradication.[3] Similar findings have also been reported in a few other studies that included CAD patients with anti *H. pylori* antibodies but without any clinical evidence of peptic ulcer disease and active *H. pylori* infection, as anti *H. pylori* antibodies themselves do not indicate whether a person is actively infected or not.[3]

Apart from its effect on fibrinogen levels, *H. pylori* could influence the development of CAD through the following mechanisms: 1. *H. pylori* can coagulate blood by stimulating mononuclear cells. Under bacterial stimulation mononuclear leukocytes produce a tissue factor like procoagulant activity, which, through the extrinsic pathway of blood coagulation, converts fibrinogen into fibrin. Thus, *H. pylori* has another activity (blood clotting) potentially contributing to CAD pathogenesis.[3] 2. Von Willebrand factor antigen is strongly associated with *H. pylori* infection, providing solid evidence that *H. pylori* positive patients have increased risk of CAD.[3] 3. *H. pylori* infection promotes formation of L - and P - selectin dependent platelet leukocyte aggregates in murine gastric microvessels and human *H. pylori* infection also induces platelet activation and aggregation. This phenomenon may be a contributory factor to the proposed relationship between *H. pylori* and CAD.[3] 4. *H. pylori* infection is associated with the increased serum levels of tumor necrosis factor α, a circulating cytokine able to exert its effects at distance. This finding sheds some light on the pathogenesis of some "extra-gastric" diseases (including CAD), which are significantly associated with *H. pylori* infection.[3] 5. Anticardiolipin antibodies, linked to CAD, have also been detected in *H. pylori* positive patients with CAD.[3] 6. Circulating concentrations of lipid peroxides also associated with cardiovascular risk, are raised in patients with *H. pylori* infection. Regarding the latter mechanism, the combination of an antioxidant with classic triple *H. pylori* eradication therapy might improve the rate of *H. pylori* eradication. Indeed, our study indicates that vitamin E improves the rate of *H. pylori* elimination without concomitant side effects.[3] Taken together, Prof. Al-Nozha could have included many of the above mentioned parameters to investigate the influence of *H. pylori* infection in the development of coronary atherosclerotic process. We believe that the lack of abnormal electrocardiogram (ECG) findings is not sufficient to support a marginal association between CAD and *H. pylori* infection in these young patients.

Moreover, the authors avoid referring to the limitations of their study in the discussion. These include: 1. Absence of a control group. 2. Absence of stratification of data according to various age groups. 3. Not clear inclusion and exclusion criteria (for example were the patients receiving antibiotics or proton pump inhibitors excluded from their study?). 4. Histology, the actual gold standard for the detection of *H. pylori* infection, did not seem to have a high accuracy in this paper. What were the stains used, and what was the accuracy of each method? Could it be that the elevated anti *H. pylori* antibodies referred to a past infection and not an active one? and 5. The cut off values that the authors used for the immunoglobulin G readings were: 155-230 u/ml positive and <135 u/ml negative. How were these values established? In our own experiments, the manufacturer's cut off value (enzyme link immunosorbent assay kit) was 10 u/ml. Were these values established in the authors' own lab and what about the values between 135 and 155 or over 230 u/ml?

Correspondence

Jannis Kountouras
Christos Zavos
Dimitrios Chatzopoulos
Department of Medicine, 2nd Medical Clinic
Ippokration Hospital
Aristotle University of Thessaloniki
8 Fanariou St., Byzantio
GR-551 33, Thessaloniki
Greece

Reply from the Author

We thank Dr. Kountouras and his colleagues for the interest and we appreciate his comments on our study.[1]

Dr. Kountouras' argument for a possible relation between *H. pylori* infection and CAD, through increasing fibrinogen level as well as other mechanisms, is not supported by a reduction in CAD after eradication of *H. pylori*.[2] A recent study showed a relationship between C-reactive protein, interleukin-6, fibrinogen, plasminogen-activator inhibitor 1 activity, D-dimer, Von Willebrand factor, plasma viscosity (collectively referred to as inflammatory markers) and seropositivity to *H. pylori*, *Chlamydia*, cytomegalovirus, as well as herpes simplex virus. However, odds for the presence of angiographically confirmed stable CAD were not increased after controlling for a variety of potential confounders.[6]

Moreover, several other investigators showed no association of CAD with *H. pylori* infection based on the following evidence that there was no relationship found between seropositivity of *H. pylori* and unstable angina or association between chronic infection with *H. pylori* and endothelial function.[7,8] Furthermore, eradication of *H. pylori* infection does not change coronary risk factors and no benefit was observed in high risk patients with CAD after treatment with antibiotics.[9] Interestingly, seropositivity for *H. pylori* was not increased in patients with acute myocardial infarction and those who tested positive for *H. pylori* were not at increased risk of atherosclerosis as indicated by measuring the mean intima media thickness of the carotid artery.[10,11]

Our study demonstrated absence of CAD in patients with proven *H. pylori* infection.[1] We agree with Dr. Kountouras that normal ECG is not sufficient to exclude CAD, however, our study was based on obtaining symptoms of CAD as well as ECG together with echocardiography, stress test, and coronary angiography. Our study, like any other retrospective study, has the limitations of absence of control group as well as lack of ability to obtain specific variables. We examined a group of patients with confirmed diagnosis of *H. pylori* for the presence of CAD that was found to be absent. The result of 0% has made no difference in stratifying patients to various groups or comparing to a control group. None of the patients included in the study was receiving antibiotics, proton pump inhibitor, or H2 blockers for at least one month prior to endoscopy. The values for serology are provided by the manufacturer and values from 135-155 u/ml are called equivocal that can be considered negative, but none of our patients had values in the equivocal range.

Recently, the finding of *H. pylori* DNA in the atherosclerotic plaques of patients with CAD has added heat to the debate of a causal relation, nonetheless, it is perhaps co-linear but not an association. Further evidences are awaited in this regard.

Mansour M. Al-Nozha
Mohamed Z. Khalil
Ibrahim Al-Mofleh
Abdullah S. Al-Ghamdi
Department of Medicine
King Khalid University Hospital
College of Medicine, King Saud University
PO Box 2925, Riyadh 11461
Kingdom of Saudi Arabia

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Correspondence

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H pylori infection and reflux oesophagitis

We read with considerable interest the paper by Kuipers *et al* (*Gut* 2004;**53**:12–20) which found no significant adverse impact on the severity of reflux disease or its control after two years of omeprazole therapy following *H pylori* eradication, during which time gastritis largely healed. In the commentary by McColl (*Gut* 2004;**53**:5–7), it is stated that although published data are conflicting, Schwizer *et al* reported improvement in reflux symptoms following *H pylori* treatment.[1] Our preliminary published data,[2] which are in accordance with those of Schwizer and colleagues,[1] consisted of a small cohort of 69 patients with gastro-oesophageal reflux disease (GORD) and irritable bowel syndrome (IBS). Forty patients were treated with omeprazole (20 mg/day) plus trimebutine (600 mg/day) for three months (group A) and 29 were treated with omeprazole (20 mg/day) as monotherapy for an equal period of time (group B). Inclusion and exclusion criteria matched those of Kuipers *et al*. Upper and lower gastrointestinal endoscopic, histological, and clinical evaluations were made at baseline. Furthermore, upper gastrointestinal evaluation was repeated three months post-treatment. At baseline, oesophagitis, confirmed by histology, and the histological presence of *H pylori* were observed in 67.9% and 62.1% and in 86% and 82.8% of groups A and B of patients, respectively. All *H pylori* positive patients received eradication treatment, as analysed in Kuipers *et al*'s paper. The eradication rate was observed in 84% of *H pylori*/positive patients in group A and in 83% of *H pylori*/positive patients in group B. Three months post-treatment, there was a significant improvement in GORD ($p = 0.003$), IBS symptoms ($p<0.0001$), and oesophagitis ($p = 0.029$) in group A compared with group B. At baseline, all 24 *H pylori*/positive patients who received omeprazole and the eradication regimen had GORD symptoms and 15 (62.5%) had histologically proven oesophagitis. Three months post-treatment, GORD was present in 12 (50%) patients ($p<0.0001$) and oesophagitis in nine (37.5%) ($p<0.05$). As improvement in oesophagitis did not reach a statistically significant level, our study was continued and results are shown in table 1 (unpublished data). All 45 *H pylori*/positive patients who received omeprazole and the eradication regimen had GORD and 29 (64.4%) had oesophagitis at baseline. Three months post-treatment, 22 (48.9%) had GORD symptoms ($p<0.00001$) and 18 (40%) had oesophagitis ($p = 0.01$) (Wilcoxon's rank sum test, two tailed p values; data not shown in table 1). There was a statistically better response in patients who also received trimebutine.

In our preliminary study, Barrett's oesophagus was observed in eight (20%) of 40 and in five (17.2%) of 29 patients in groups A and B, respectively, similar to that (24.3%) observed by Kuipers *et al*. As there is an increased prevalence of IBS in patients with GORD,[3] it would be interesting to know how many of the patients in Kuipers *et al*'s study had symptoms suggestive of IBS, and if their regimen had results similar to ours.

Our data show that *H pylori* is frequent in GORD and may contribute to the pathogenesis of GORD by several mechanisms.[4] Also, we propose that the increasing prevalence of GORD may be partially explained not just by the decrease in prevalence of *H pylori* infection, as suggested by McColl (*Gut* 2004;**53**:5–7), but rather by healing of *H pylori* associated peptic ulcer disease, which coexists with GORD.[5 6] Thus eliminating peptic ulcer disease unmasks GORD.[5]

In our latest unpublished data, 18 (48.6%) of 37 patients, in whom *H pylori* was eradicated, had reflux symptoms on omeprazole compared with four (50%) of eight patients in whom *H pylori* was not eradicated. Although the latter group was too small to draw definite conclusions, it seems that eradicating *H pylori* did not make GORD more difficult to control. While the editorial advocates that *H pylori* eradication makes it more difficult to achieve long term control of GORD with omeprazole therapy, we suggest that *H pylori* eradication leads to better control of GORD symptoms and improves oesophagitis.

J Kountouras, C Zavos, D Chatzopoulos
Department of Medicine, Second Medical Clinic, Aristotle University of Thessaloniki, Ippokration Hospital, Thessaloniki, Greece

Correspondence to: Dr J Kountouras, 8 Fanariou St, Byzantio, 551 33, Thessaloniki, Macedonia, Greece; jpanid@med.auth.gr

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Table 1 Patient profile prior to and after the therapeutic regimens and comparisons between the two groups

	Group A (n = 92) (omeprazole plus trimebutine)					Group B (n = 56) (omeprazole)					Significance (p)			
	Prior		After 3 months			Prior		After 3 months			A v B		A1b v B1b	A2 v B2
	A1 (n = 73)	A2 (n = 19)	A1a (n = 12)	A1b (n = 61)	A2 (n = 19)	B1 (n = 45)	B2 (n = 11)	B1a (n = 8)	B1b (n = 37)	B2 (n = 11)	Prior	After	After	After
GORD symptoms	73	19	5	10	3	45	11	4	18	7	NS	<0.001	<0.005	<0.025
Oesophagitis	51	12	5	10	2	29	7	2	16	5	NS	<0.01	<0.01	NS
IBS symptoms	73	19	2	7	3	45	11	4	23	7	NS	<0.001	<0.001	<0.025

p values between the two groups were calculated using the χ^2 test with Yates' correction (two tailed p values).
Group A, patients were assigned to three months of omeprazole plus trimebutine treatment; group B, patients were assigned to three months of omeprazole monotherapy; groups A1, B1, *H pylori* positive patients prior to treatment assigned to omeprazole plus trimebutine or omeprazole alone, respectively; groups A1a, B1a, patients from groups A1 and B1, respectively, who remained *H pylori* positive after the eradication regimen; groups A1b, B1b, patients from groups A1 and B1, respectively, in whom *H pylori* infection was eradicated; groups A2, B2, *H pylori* negative patients prior to and after treatment with omeprazole plus trimebutine or omeprazole alone, respectively

Correspondence

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Salt intake and *Helicobacter pylori* infection

Jannis Kountouras, Christos Zavos and Dimitrios Chatzopoulos

Department of Medicine, Second Medical Clinic, Aristotle University of Thessaloniki, Ippokration Hospital, Thessaloniki, Greece.

Correspondence and requests for reprints to Jannis Kountouras, 8 Panariou Street, Ilyssia, 551 33, Thessaloniki, Macedonia, Greece.
Tel: +30 2310 892236; fax: +30 2310 992794; e-mail: jannis@med.auth.gr

We read with considerable interest the paper by Beevers *et al.* [1] and the editorial comment by Gueyffier [2]. One hypothesis advanced is that a high-salt diet irritates the gastric mucosa and allows *Helicobacter pylori* to enter the body. However, *H. pylori* is considered to be an extracellular micro-organism and many studies on human gastric biopsy specimens have failed to demonstrate any intracellular *H. pylori*; this bacterium lies on the surface of the gastric mucosa and adheres intimately to gastric epithelial cells with specific tissue tropism, thereby triggering several mechanisms that lead to damage of the gastric mucosa. Although, for many other enteric bacterial infections, epithelial cell invasion is of major significance for the survival and multiplication of the micro-organisms, this is not the rule for *H. pylori* infection, for which invasion of epithelial cells by these bacteria appears to be rare. Therefore, the hypothesis that salt intake facilitates *H. pylori* invasion by inducing gastric damage appears unlikely.

This bacterium could influence the pathophysiology of hypertension by one of several mechanisms [3–5]. (i) Under bacterial stimulation, mononuclear leukocytes produce a tissue factor-like procoagulant activity, which, through the extrinsic pathway of blood coagulation, converts fibrinogen into fibrin. Thus, *H. pylori*, possessing blood clotting activity, contributes to hypertension pathogenesis and complications; hypertension abnormalities in plasma prothrombotic factors and markers of endothelial dysfunction are consistent with the increased risk of thrombotic events (strokes, heart attacks) in hypertensive patients. In this regard, our study demonstrates that increased fibrinogen levels (a marker of vascular damage that contributes to the evolution of the complications of hypertension) are associated with *H. pylori* infection and can be significantly reduced by bacterial eradication [5]. (ii) Von Willebrand factor antigen is strongly associated with *H. pylori* infection, providing solid evidence that *H. pylori*-positive patients have increased risk of hypertension. Notably, von Willebrand factor (an endothelial damage index) is associated with hypertension.

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(iii) Human *H. pylori* infection induces oxidative stress and platelet activation and aggregation [3,4], which, in turn, contribute to development of hypertension and promotion of vascular damage. Oxidative stress is known to induce apoptosis and activation of nuclear factor-κB, whose biological effects may influence the *H. pylori* infection and arterial hypertension sequelae. In this regard, the Fas-Fas ligand apoptotic system and high-density lipoprotein-cholesterol (found to be decreased in our *H. pylori*-infected patients) are associated with atherosclerosis in hypertensive patients. (iv) *Helicobacter pylori* infection is associated with increased serum tumour necrosis factor (TNF)-α (the gene of TNF-receptor SF1B has been implicated in hypertension) and other pro-inflammatory cytokines (interleukins 1 and 6) or eicosanoids involved in a number of vascular *H. pylori*-associated 'extra-gastric' disorders, including systemic hypertension [4]. (v) Regulation of blood pressure depends on vasoactive agents such as nitric oxide and endothelin (ET)-1 (a potent arteriole and venule constrictor) [4]. Elevated ET-1 levels in hypertensive and *H. pylori*-infected patients may contribute to systemic vasoconstriction, indicating vascular dysfunction.

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The beneficial effect of β_2-blockers on humoral autoimmune disorders

Mohammed R. Namazi

Dermatology Department, Shiraz University of Medical Sciences, Shiraz, Iran.

Correspondence and requests for reprints to Dr M. R. Namazi, PO Box 71955–687, Shiraz, Iran.
E-mail: namazi_md@yahoo.com

It has been shown that lymphocytes and macrophages express β_2-receptors, and that stimulation of these receptors with catecholamines leads to inhibition of cell-mediated (or type 1) immunity and stimulation of immunoglobulin production and the type 2 immune

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Reprint requests and correspondence: Andrew Mason, Division of Gastroenterology, Suite 205, College Plaza 8215–112th St. Edmonton, AB, Canada AB T6G 2C8 e-mail: andrew.mason@ualberta.ca

Accuracy of E-test for Metronidazole Susceptibility in *H. Pylori*

Dear Sir,

We are very grateful for the interest Dr. Osato and Graham (1) showed in our work (2). We would like to point out a few points. (1) We start from an inoculum size of approximately 108 CFU/ml as recommended by the NCCLSS-approved agar dilution method. With fast-growing bacteria, such a concentration corresponds to the opacity standard McFarland 0.5 (1.5 × 108, to be correct). With *H. pylori*, in order to obtain 108 vital organisms per ml, you have to suspend the biomass up to McFarland opacity standard # 4. (2) You cited as example the result published by Megraud *et al*. (3), and we agree with you, but, unfortunately, they used an opacity standard of McFarland # 3, prepared in brucella broth from 48-h agar plate cultures and read after 48 h, instead of McFarland # 2 prepared in a saline solution from a 72-h agar plate and read after 72 h, described in NCCLS procedures; is that work wrong? (3) Anyway, preliminary tests assessed in about 20 strains showed no discrepancies in the pattern of susceptibility between inocula corresponding to McFarland # 2 and # 4. (4) We showed, together with other authors (4, 5), that the reliability of E-test is linked to the presence of mixed infection, and the different geographic prevalences of bacterial subpopulation as well as the increase of mixed infection in the treated patients are well known. So the different results published in the literature may be due to these two factors. In fact, we assessed the presence of mixed infection in 13.9% of the isolated strains, while this percentage ranged from zero to 80% in other studies. (5) Moreover, we specified that the strains tested for the study were isolated from patients who had never been treated. Finally, wan der Wouden (4), Piccolomini (6), Glupczynski (7), and Hirschl (8) found a good correlation between agar dilution and E-test, so we think that when a lot of "small" studies show similar results they have to be considered.

*Perna, Ph.D.**
*Figura, M.D.***
*Gatta, M.D.**
*Ricci, M.D.**
*Bernabucci, M.D.**
*Volpe, M.D.**

**Department of Internal Medicine and Gastroenterology University of Bologna, Italy*
***Department of Internal Medicine Siena, Italy*

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Induction of Interleukin-8 Expression by *Helicobacter Pylori* Infection in Patients with Endoscopy-Negative Gastroesophageal Reflux Disease

TO THE EDITOR: We read with considerable interest the article by Isomoto *et al*. (1). The authors do not comment on *Helicobacter pylori* infection (*Hp-I*), which appears to influence the pathophysiology of gastritis and probably gastroesophageal reflux disease (GERD), mainly through the induction of interleukin (IL)-8 (2). Our initial data indicate that *Hp* is frequent in Greek patients with endoscopy-positive

GERD and may contribute to GERD pathogenesis by several mechanisms (3, 4). In a subsequent study (5), we investigated 31 patients with endoscopy-negative reflux disease (NERD). In 25 of 31 (80.64%) patients, histologic evidence of esophagitis (neutrophil/monocyte inflammatory infiltrations) was detected, and histologic presence of *Hp-I* was found in gastric mucosa in 13 of 25 (52%) patients, whereas only 1 of 6 (16.67%) patients without inflammatory cell infiltrations was *Hp* infected. Therefore, the histologic presence of esophagitis with concomitant *Hp-I* seems to be common in NERD Greek patients.

Importantly, *Hp-I* is associated with increased expression and production of C-X-C chemokine IL-8, and triggers an intense leukocyte infiltration mediated by other proinflammatory cytokines (tumor necrosis factor (TNF), IL-1, and IL-6 from human mononuclear cells) (2). In particular, increased IL-8 immunoreactivity and increased IL-8 mRNA expression in *Hp*-infected mucosa have been detected *in vivo*, and the IL-8 expression is upregulated by the inflammatory cytokines (TNF-α IL-1) produced in *Hp*-infected upper GI tract mucosa (2). Inflammatory cytokines are increased in patients infected with strains of the CagA phenotype. CagA is not the direct inducer of IL-8 but CagA positive strains are associated with increased gastric IL-8 mRNA expression and IL-8 protein *in vivo*. IL-8 secretion from neutrophils following stimulation with *Hp* lipopolysaccharide (*Hp*-LPS) is lower than that induced by *Escherichia coli* and *Salmonella* LPS. This low biological activity of *Hp*-LPS, a result of the phosphorylation pattern of its lipid A component may prolong *Hp-I*, thereby contributing to chronic inflammation in the gastric and probably esophageal mucosa (1). Moreover, *Hp* induces LPS-independent soluble surface proteins with chemotactic activities to recruit neutrophils and monocytes to the lamina propria and to activate these inflammatory cells. These proteins include *Hp* neutrophil-activating protein (*Hp*-NAP, 150 kDa) and the immunologically active porins. The napA gene, encoding the *Hp*-NAP, is present in all *Hp* strains (1). Apart from IL-8, the gastric and probably esophageal epithelium secretes other neutrophil attractant chemokines (GROα), in response to *Hp-I* (1). Finally, the expression of C-X-C chemokine decreases significantly in patients after successful *Hp* eradication (2), findings in accordance with our results showing improvement in GERD symptoms and esophagitis after *Hp* eradication (4).

Jannis Kountouras, M.D., Ph.D.
Christos Zavos, M.D.
Dimitrios Chatzopoulos, M.D.

Department of Medicine, Second Medical Clinic
Aristotle University of Thessaloniki
Ippokration Hospital
Thessaloniki, Greece

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Reprint requests and correspondence: Jannis Kountouras, M.D., Ph.D., Associate Professor of Medicine Gastroenterologist, 8 Fanariou St, Byzantio 551 33, Thessaloniki, Macedonia, Greece; e-mail: jannis@med.auth.gr

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Expression of Interleukin 8 Messenger Ribonucleic Acid in Esophageal Mucosa of Patients with Gastroesophageal Reflux Disease in Terms of *Helicobacter pylori* Status: Response

TO THE EDITOR: We would like to thank Dr. Kountouras *et al*. for their interest in our work (1). They raise the possibility that *Helicobacter pylori* infection may influence the pathophysiology of gastroesophageal reflux disease (GERD) mainly through induction of interleukin (IL)-8. First, *H. pylori* infection rates were 32.3% (10/31) and 33.3% (5/15) in patients with endoscopy-negative GERD and erosive esophagitis, respectively (1). These were much lower compared to the prevalence in patients without GERD who underwent upper endoscopy in our hospital (2). Second, we studied the expression levels of IL-8 messenger ribonucleic acid (mRNA) levels in esophageal mucosa in the same study population with reference to *H. pylori* infection, employing a real-time quantitative polymerase chain reaction procedure (1). There was no difference in the relative IL-8 mRNA expression levels, expressed as the ratio of IL-8/tubulin alpha 3 (housekeeping gene) in arbitrary units, between GERD patients with and without *H. pylori* infection (1.65 ± 2.93 *vs* 1.31 ± 1.15). Thus, it is unlikely that *H. pylori* status may affect IL-8 mRNA expression in esophageal mucosa of GERD patients. Apart from IL-8, the expression of such chemokines as monocyte chemoattractant protein-1 and regulated on activation normal T-cell expressed and presumably

secreted is significantly more elevated within esophageal mucosa of subjects with GERD than those without GERD (3). There is, however, little information on the implication of *H. pylori* infection in diverse chemokines and proinflammatory cytokines produced locally in GERD.

We have not assessed the IL-8 mRNA expression levels in esophageal mucosa before and after *H. pylori* eradication. The effect of *H. pylori* eradication in patients with GERD remains unclear; some investigators suggest that this might be counterproductive and that *H. pylori* infection might protect against the disease (4), whereas others reported improvement of GERD symptoms and esophagitis after cure of the infection (5). Clearly, well-designed, large-scale, case-control studies are warranted to unravel the complex relation between *H. pylori* eradication and GERD development.

Hajime Isomoto, M.D.
Yasei Kanazawa, M.D.
Shigeru Kohno, M.D., Ph.D.
Second Department of Internal Medicine
Nagasaki University School of Medicine
Nagasaki, Japan

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Reprint requests and correspondence: Hajime Isomoto, M.D., Gastrointestinal Unit, Massachusetts General Hospital, Department of Medicine, Harvard Medical School, Jackson 706, 55 Fruit Street, Boston, MA 02114.

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Early Intensive Resuscitation of Patients with Upper Gastrointestinal Bleeding Decreases Mortality

TO THE EDITOR: The recent article by Baradarian *et al.* and colleagues carried an important message for the optimum management of upper gastrointestinal bleeding (1). However, I am concerned about the statistical methods used and their conclusion.

Their result showed that the intensive resuscitation group had in-patient mortality of one patient (2.8%) compared to four patients (11%) in the observational group. Chi-squared test of this categorical data showed a statistical significant difference between the two groups with a *p*-value of 0.04. This result is incorrect. Fisher's exact test should be the correct hypothesis test instead of the χ^2 test because the expected (E) frequency is 2.5 in two out of four cells (2).

	Outcome		
Group	Alive (E)	Died (E)	Total
Intensive	35 (33.5)	1 (2.5)	36
Observation	32 (33.5)	4 (2.5)	36
Total	67	5	72

The two-sided *p*-value was 0.357. I was also unable to reproduce their results using the χ^2 test. Hence, the study cannot reject the null hypothesis that there is no difference in outcome between the two groups. I suspect the study is underpowered to detect a difference in mortality between the two groups because no power calculation was done. I recommend a correction of their results and conclusion.

Chee H. Lim, M.B.Ch.B.

Centre for Digestive Disease
Leeds General Infirmary
LEEDS LS1 3EX, United Kingdom

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Reprint requests and correspondence: Dr. Chee H. Lim, Gastroenterology Dept., Room 190A, Clarendon Wing, Leeds General Infirmary, Leeds LS1 3EK, United Kingdom.

Response to Dr. Lim

TO THE EDITOR: We found the comment by Dr. Lim regarding our statistical methods as important. Our comparative study evaluating patients with upper gastrointestinal bleeding showed a decreased morbidity and mortality in patients treated with "intensive resuscitation" (1). We concede that a Fisher exact test would have been the preferred method of evaluating the differences between the two groups. Also, the χ^2 part of the statistic utilized in our study is 0.04, not the *p*-value. As correctly pointed out by Dr. Lim, the *p*-value is