ΞΕΝΟΓΛΩΣΣΕΣ ΔΗΜΟΣΙΕΥΣΕΙΣ ΕΛΛΗΝΩΝ ΕΡΕΥΝΗΤΩΝ

(με αλφαβητική σειρά κατά συγγραφέα)

Scandinavian Journal of Gastroenterology, 2008; 43: 922–928

informa
healthcare

ORIGINAL ARTICLE

Patients with duodenal ulcer have lower levels of serum cholesterol compared to other dyspeptic patients independently of *Helicobacter pylori* status

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Abstract

Objective. The association between *Helicobacter pylori* (*H. pylori*) infection and serum lipid profile is still controversial. The aim of this study was to determine any possible relationship between *H. pylori* infection and the lipid profile of patients with upper gastrointestinal symptoms. ***Material and methods.*** Consecutively selected 20–70 year-old dyspeptic patients who had undergone esophagogastroduodenoscopy were evaluated for *H. pylori* infection using both the CLO test and Giemsa staining. Serum total cholesterol (C), HDL-C, LDL-C, apo-A_1, apo-B and triglyceride levels were measured. ***Results.*** A total of 137 patients (median age 52.0 years) were studied. Total cholesterol levels were lower in *H. pylori*-infected patients than in *H. pylori*-negative patients (mean $\pm$SEM: 199.3 $\pm$ 5.9 versus 212.6 $\pm$ 4.6 mg/dl, $p = 0.08$). Patients with duodenal ulcer (DU) had significantly lower levels of all measured lipidemic parameters including cholesterol, with the exception of triglycerides, in comparison with either *H. pylori*-positive or -negative dyspeptic patients (cholesterol: 177.6 $\pm$ 6.5 versus 214.6 $\pm$ 4.2 mg/dl, $p < 0.0001$). However, there was no difference in the total cholesterol/HDL-C ratio between DU patients and the rest of the dyspeptic patients. ***Conclusions.*** Among *H. pylori*-positive and *H. pylori*-negative patients there was no difference in lipid profile apart from a trend towards total cholesterol levels being lower in *H. pylori*-positive patients. However, cholesterol, HDL-C, LDL-C, apo-A and apo-B were all decreased in DU patients even though this reduction did not result in a fall in the total cholesterol/HDL-C ratio. The etiologic factor differentiating the lipid profiles among dyspeptics only in *H. pylori*-positive patients carrying a DU could be dietetic, microbial, genetic or a combination of all three.

Key Words: *Apolipoprotein, cholesterol, duodenal ulcer, HDL, Helicobacter pylori, LDL, triglycerides*

Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium that commonly causes chronic inflammation of the gastric mucosa. Even thought the majority of infected individuals are asymptomatic, the infection is associated with peptic ulcer, gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma [1–4]. Furthermore, a potential role of *H. pylori* infection in several extra-digestive diseases has been reported, suggesting that different strains of this bacterium are correlated to different biological activities [5–8].

There is some evidence to show an association between *H. pylori* infection and serum lipid profile. This could be important, as an altered lipid profile is a risk factor for cardiovascular diseases [9]. However, the data regarding this subject are controversial; some investigators have reported an influence of *H. pylori* infection on serum lipid pattern [10–13], while others have found no association [14–16]. Nevertheless, all but two of these studies were based on either serum anti-*H. pylori* antibody measurements for identification of *H. pylori*-infected patients or the urea breath test. However, the measuring of

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(Received 4 December 2007; accepted 19 February 2008)

ISSN 0036-5521 print/ISSN 1502-7708 online
DOI: 10.1080/00365520802008132

serum anti-*H. pylori* antibodies in order to evaluate active *H. pylori* infection has been shown to have reproducibility problems and cannot accurately reflect a true infection [17]. Moreover, given that there was no esophagogastroduodenoscopy (EGD) available in these studies, there is a paucity of data on the existence of peptic ulcer and its possible interaction on serum lipid profile. Owing to the scarcity of information, we conducted this study in order to investigate the relationship between *H. pylori* infection and serum lipid pattern, taking into account the presence of active *H. pylori* infection as well as the presence of peptic ulcer as a potential confounder.

Material and methods

Consecutive dyspeptic patients, ranging in age from 20 to 70 years, who underwent EGDs were prospectively studied in order to determine any relationship between *H. pylori* infection and lipid profile. Patients were recruited over a 3-month period (April–June 2006). Those with a history of eradicated *H. pylori* infection, suspected or confirmed malignancy, diabetes mellitus, thyroid, liver, renal and coronary heart diseases or receiving antilipidemic treatment were excluded. Consumption of proton-pump inhibitors or histamine-2 receptor antagonists for more than 3 consecutive days in the past month or aspirin and/or non-steroidal anti-inflammatory drugs (NSAIDs) in the past 2 weeks, and regular alcohol consumption were also defined as exclusion criteria. Furthermore, at endoscopy, we excluded all patients with current complications of ulcer disease (pyloric stenosis, active bleeding or perforation).

The recorded epidemiological characteristics were gender, age, body mass index (BMI) and smoking. Lipid profile consisted of total serum cholesterol (CHOL), high-density lipoprotein (HDL)-cholesterol (HDL-C), low-density lipoprotein (LDL)-cholesterol (LDL-C), triglycerides (TGs), apolipoprotein-A (apo-A) and apolipoprotein-B (apo-B). Furthermore, we evaluated the classic ratio of CHOL to HDL-C, which is widely used as an indicator of lipid-related coronary risk.

H. pylori infection was evaluated by rapid urease test (CLO test; Ballard Medical Products, Draper, Utah, USA) using a single antral specimen and by histological examination with modified Giemsa stain of four (two antral and two corporal) biopsies. Patients were considered to be *H. pylori* positive if both the rapid urease test and the histology were positive.

Serum concentrations of CHOL, HDL-C, LDL-C and TG were measured using the standard enzymatic colorimetric method, while apo-A and apo-B were measured by immunoturbidimetry (Roche Diagnostics GmbH, Mannheim, Germany). The blood sample for determination of the lipid profile of each participating patient was collected a few minutes before the EGD procedure, as part of the standard laboratory analysis.

The study protocol was approved by the ethics committee of our hospital and written informed consent was obtained from all of the patients.

Statistical analysis

Data were summarized as mean values ± standard error of the mean. The baseline characteristics of the groups (infected and non-infected by *H. pylori*) were compared using Student's unpaired two-tailed *t*-test for the means and the χ^2 test for the proportions. Logistic regression models were used to examine the association between variables identified in terms of odds ratios (ORs) with 95% confidence intervals (95% CI) and Hosmer-Lemeshov goodness-of-fit tests were used for model checking. Multiple regression models included all variables found to have a *p*-value of less than 0.1 in a univariate analysis, as well as all known confounding factors (gender, age and smoking). In a multivariate analysis, a *p*-value of less than 0.05 was considered to indicate statistical significance and reported *p*-values were two sided.

Results

A total of 137 patients (M 64, F 73, age range 20–70 years, median age 52.0 years) fulfilled the inclusion criteria and were enrolled in the study, but 4 of them were excluded from the analysis owing to discordance in the results between the CLO test and Giemsa staining. The final study population consisted of 133 adult patients, of whom 66 (49.6%) were *H. pylori* positive and 67 (50.4%) *H. pylori* negative.

There were no significant differences in the demographic variables (age, gender, BMI, smoking) between patients with *H. pylori* infection and those without; nor were there any significant differences in LDL-C, HDL-C, TGs, apo-A, apo-B levels and CHOL/HDL-C ratio between the two groups, but the difference in CHOL levels tended to be significant ($p = 0.08$, Table I). Specifically, for each 10 mg/dl increase in CHOL level, patients were 7% less likely to be infected by *H. pylori* (OR: 0.93, 95% CI 0.86–1.008, $p = 0.08$, data not shown).

Thirty-one patients were found to have duodenal ulcers (DU), but no gastric ulcers were found, probably because of the exclusion criteria of NSAIDs and aspirin consumption. When adjusting for age, gender, smoking and DU using multiple

Table I. Demographic and serum lipid profile characteristics of the study population according to *H. pylori* status.

Characteristics	*H. pylori* negative ($n=67$)	*H. pylori* positive ($n=66$)	*p*-value
Age (years)	50.8±1.6	49.6±1.7	NS
Gender: Male (N%)	28 (41.8)	34 (51.5)	NS
BMI (kg/m^2)	25.1±0.6	25.7±0.6	NS
Duodenal ulcer (N%)	4 (6.0)	27 (40.9)	<0.0001
Smoking (N%)	22 (32.8)	26 (39.4)	NS
Total cholesterol (mg/dl)	212.6±4.6	199.3±5.9	0.08
Triglycerides (mg/dl)	110.6±6.3	102.9±6.9	NS
LDL-C (mg/dl)	142.9±4.2	134.7±5.1	NS
HDL-C (mg/dl)	56.2±2.0	53.1±1.8	NS
Apo-A (mg/dl)	149.3±3.2	142.1±3.2	NS
Apo-B (mg/dl)	99.2±2.9	94.1±3.3	NS
CHOL/HDL-C ratio	4.1±0.2	3.9±0.2	NS

Abbreviations: BMI = body mass index; LDL-C = low-density lipoprotein-cholesterol; HDL-C = high-density lipoprotein-cholesterol; NS = not significant.

logistic regression analysis, the difference in CHOL levels between the two groups of patients (with or without *H. pylori*) was no longer statistically significant. This was due to a strong confounding effect of the presence of DU (Table II).

Characteristics of the study population concerning the presence of DU are presented in Table III. As expected, we found a higher prevalence of *H. pylori* infection ($p<0.0001$), cigarette smoking habit ($p=0.04$) and male gender ($p=0.002$) among patients with DU. In addition, patients with DU were younger than those without DU ($p=0.01$). However, in the multiple regression analysis, there was no association between age and presence of DU. Regarding CHOL, the levels were significantly lower in patients with DU even after adjustment for potential confounding factors (age, gender, smoking, *H. pylori* infection) – (Table IV). For each 10 mg/dl increase in CHOL levels, patients were 18% less likely to have DU (OR: 0.82, 95% CI 0.72–0.95, $p=0.006$, data not shown). Moreover, LDL-C and apo-B levels were also significantly lower in patients with DU (OR: 0.98, 95% CI 0.96–0.99, $p=0.005$ and OR: 0.97, 95% CI 0.95–0.99, $p=0.02$, respectively, data not shown). Conversely, CHOL/HDL-C ratio (Table V), HDL-C and apo-A were not found to be associated with DU (data not shown).

Table II. Association of *H. pylori* with patients' characteristics.

Characteristics	*n* (%)	Adjusted OR† (95% CI) for *H. pylori* (+)
Total cholesterol (mg/dl)	133 (100)	0.99 (0.99–1.00)
Age (years)	133 (100)	1.01 (0.98–1.04)
Gender		
Female	71 (53.4)	1.0
Male	62 (46.6)	0.94 (0.43–2.07)
Smoking		
No	85 (63.9)	1.0
Yes	48 (36.1)	1.07 (0.46–2.51)
Duodenal ulcer		
No	102 (76.7)	1.0
Yes	31 (23.3)	11.5* (3.40–39.22)

Abbreviation: OR = odds ratio.
†The model includes total cholesterol, age, gender, smoking and duodenal ulcer. Therefore, the ORs reported are adjusted for other variables; *$p \leq 0.0001$.

Discussion

It has been suggested that *H. pylori* infection constitutes a risk factor for coronary heart disease by affecting lipid metabolism [5,12]. However, the association between *H. pylori* infection and serum lipids remains unclear. Several studies yield various and sometimes contradictory results; increased, normal and decreased level of lipids, all being reported [10–16].

The diagnosis of *H. pylori* infection in these studies was based on various methods, either non-invasive or invasive, with advantages and disadvantages in each case. In our study, *H. pylori* infection was considered to be present if both the histological examination and the rapid urease test were positive. Those strict criteria increased the accuracy of diagnosis of active *H. pylori* infection compared with serology. The latter has a low diagnostic accuracy (80–84%), is unable to detect active infection and could result in misinterpretation owing to limits in test reproducibility [17,18]. Besides the increased false-positive results of non-invasive tests in populations with a low *H. pylori* prevalence, serological tests are recommended when assessing infection in patients with a bleeding ulcer and conditions associated with low bacterial density (extensive mucosal atrophy and MALT lymphoma) [18]. The rapid urease test has a satisfactory accuracy (90%), especially in patients who are not

Table III. Characteristics of study population concerning the presence of duodenal ulcer.

Characteristics	Patients with DU ($n=31$)	Patients without DU ($n=102$)	p-value
Age (years)	44.8±2.9	51.9±1.2	0.01
Gender (N%) Male	22 (71.0)	40 (39.2)	0.002
BMI (kg/m^2)	24.9±0.8	25.5±0.5	NS
H. pylori (N%)	27 (87.1)	39 (38.2)	<0.0001
Smoking (N%)	16 (51.6)	32 (31.4)	0.04
Total cholesterol (mg/dl)	177.6±6.5	214.6±4.2	<0.0001
Triglycerides (mg/dl)	108.4±9.7	106.3±5.6	NS
LDL (mg/dl)	117.3±5.7	145.4±3.7	0.0003
HDL (mg/dl)	48.3±2.2	56.6±1.6	0.009
Apo-A (mg/dl)	135.4±4.3	148.9±2.6	0.01
Apo-B (mg/dl)	86.1±3.3	99.9±2.6	0.007

Abbreviations: BMI = body mass index; DU = duodenal ulcer; LDL = low-density lipoprotein; HDL = high-density lipoprotein; NS = not significant.

taking antibiotics, antisecretory drugs or who have had recent upper gastrointestinal bleeding, conditions that were excluded in our cohort, in accordance with the protocol. It is suggested that two biopsies, one from the antrum and one from the corpus, are better than one in order to increase the sensitivity of the urease test when evaluating *H. pylori* eradication [19]. However, the advantage of taking two biopsies in naïve patients is less clear; we evaluated our patients using only an antral specimen taken 3 cm from the pylorus, where the probability of *H. pylori* detection is maximal [20]. The accuracy of histology depends on the size, number and the site of biopsies; under optimal conditions this is considered high (90%) [21]. In order to increase the sensibility of histology, we obtained four biopsies, two antral biopsies 3 cm from the pylorus and two corporeal biopsies from the median part of the greater curvature. Although concordance between pathologists is not perfect for *H. pylori* gastritis evaluation, the best agreement is reached in the assessment of *H. pylori* density [22]. We excluded 4 out of 137 patients because of a disagreement between the histology and the CLO test in order to increase the accuracy of the *H. pylori* positivity. Another advantage of our study protocol was endoscopy, which is absent in most of the previous studies negotiating the correlation between *H. pylori* and

Table IV. Association of duodenal ulcer with patients' characteristics, including total cholesterol.

Characteristics	n (%)	Adjusted OR† (95% CI) for DU (+)
Total cholesterol (mg/dl)	133 (100)	0.98** (0.96–0.99)
Age (years)	133 (100)	0.98 (0.95–1.02)
Gender		
Female	71 (53.4)	1.0
Male	62 (46.6)	3.08*** (1.08–8.78)
Smoking		
No	85 (63.9)	1.0
Yes	48 (36.1)	2.64 (0.85–8.22)
H. pylori		
No	67 (50.4)	1.0
Yes	66 (49.6)	9.81* (2.94–32.71)

Abbreviations: OR = odds ratio; DU = duodenal ulcer.
†The model includes total cholesterol, age, gender, smoking and *H. pylori* infection. Therefore, the ORs reported are adjusted for the other variables.
*$p \leq 0.0001$; **$p \leq 0.01$; ***$p \leq 0.05$.

Table V. Association of duodenal ulcer with patients' characteristics including total cholesterol/HDL-cholesterol ratio.

Characteristics	n (%)	Adjusted OR† (95% CI) for DU (+)
Ratio (CHOL/HDL-C)	133 (100)	0.70 (0.46–1.05)
Age (years)	133 (100)	0.97 (0.94–1.00)
Gender		
Female	71 (53.4)	1.0
Male	62 (46.6)	4.67** (1.63–13.33)
Smoking		
No	85 (63.9)	1.0
Yes	48 (36.1)	2.24 (0.77–6.46)
H. pylori		
No	67 (50.4)	1.0
Yes	66 (49.6)	11.22* (3.40–36.96)

Abbreviations: CHOL = total serum cholesterol; HDL-C = high-density lipoprotein-cholesterol; OR = odds ratio; DU = duodenal ulcer.
†The model includes cholesterol/HDL-cholesterol ratio, age, gender, smoking and *H. pylori* infection. Therefore, the ORs reported are adjusted for the other variables.
*$p \leq 0.0001$; **$p \leq 0.01$.

lipids. Endoscopy permitted the evaluation of both gastroduodenal lesions and *H. pylori*. The urea breath test, which is a more accurate non-invasive method than serology for the detection of active *H. pylori* infection, cannot identify patients with peptic ulcer, as is the case with all non-invasive methods.

In order to homogenize our study population, we excluded patients with a history of *H. pylori* eradication treatment. However, our results could not be projected to the general population, as our study consisted of patients with dyspeptic symptoms. Additionally, the high prevalence of DU patients in our study population was probably related to the type of recruitment of this population; a mixed system of referrals to our tertiary center hospital and open-access consultation activity. Nevertheless, the dichotomy between patients with and those without DU in *H. pylori*-infected patients in our study permitted disclosure of the differences in their lipidemic profiles, irrespectively of the DU prevalence in the general population.

The rationale for changes expected to be found in the lipid profile in *H. pylori*-infected individuals is related to the altered lipid metabolism manifested in the acute-phase response induced by infections [23]. Inflammatory mediators such as INF-γ, IL-12, IL-18, IL-17 and TNF-α involved in acute-phase responses are similar to those involved in responses of the gastric mucosa to *H. pylori* infection [24]. However, different factors related to genetics, age, gender, diet, environment and infection can influence the type of host gastric immune responses and disease expression. The discrepancy in the results correlating *H. pylori* and lipid profile observed in previous studies could be attributed to the heterogeneous responses and disease expression in the gastric mucosa. In our study, we found a decrease only in CHOL levels among the serum lipid profile parameters measured in the univariate analysis, which tended to be significantly lower in *H. pylori*-infected patients than in the *H. pylori*-negative patients ($p = 0.08$). There were no statistical differences in the rest of the lipid profile (TG, HDL-C, LDL-C, CHOL/HDL-C ratio, apo-A and apo-B) between the two groups. In the multiple regression models, the difference in CHOL levels between the *H. pylori*-positive and *H. pylori*-negative groups of patients lost any statistical significance because of the strong confounding effect of DU.

When DU patients were evaluated as a separate subgroup (Table III), CHOL as well as HDL-C, LDL-C, apo-A and apo-B levels were lower in this group compared with in patients without DU, irrespectively of *H. pylori* status. When patients with DU but without *H. pylori* infection (4 patients) were excluded from this subgroup, the above-mentioned differences remained unchanged. Considering that favorable and unfavorable parameters for cardiovascular risk, such as CHOL, HDL-C, LDL-C, apo-A and apo-B, were all decreased in DU patients, we evaluated the CHOL/HDL-C ratio [25]. We found no reduction in this ratio even after adjustment for all potential confounding factors (Table IV). In an intervention study, Elizalde et al. found no difference in the lipid profile at baseline characteristics between *H. pylori*-positive and *H. pylori*-negative patients after adjusting for age, gender, smoking status and endoscopic diagnosis [16]. However, the results of endoscopy showed the presence or absence of peptic ulcer without elucidating whether it concerned the duodenum or the stomach. In contrast, our population consisted solely of DU patients, thus forming a more homogeneous group. When they used the urea breath test, Hoffmeister et al. found a decrease in HDL-C as well as in the HDL-C to total CHOL ratio in *H. pylori*-positive healthy volunteers compared with *H. pylori*-negative volunteers [13]. This difference was not found in patients with coronary heart disease. The lack of an endoscopic examination deprived this study of the opportunity to consider DU as a possible independent factor. In a recent study examining an asymptomatic Korean population, it was concluded that TGs, HDL-C and apolipoproteins were decreased in *H. pylori*-positive patients, independently of the presence of peptic ulcer [26]. This is in contrast to our results in dyspeptic patients. However, one should be aware that the prevalence of peptic ulcer in the Korean study is not well defined. Nevertheless, its influence must be low because the population examined was asymptomatic.

Theoretically, the difference in CHOL levels could be attributed either to dietary modifications due to DU and its related symptoms or to the *H. pylori* strain related to DU [27,28]. In addition, this effect could be influenced by genetic factors contributing to the susceptibility in *H. pylori*-positive patients for DU formation. *H. pylori* infection produces high levels of TNF and other pro-inflammatory cytokines in the gastric mucosa [29]. TNF-α possesses a broad range of activities in initiating and amplifying inflammation. It is well known that TNF-α gene polymorphisms have been implicated as potential determinants of disease susceptibility [30,31]. *H. pylori* infection can lead to superficial gastritis and DU with high acid secretion, or to atrophic gastritis and gastric cancer with low acid secretion. These differences have been correlated to *H. pylori* strains of different virulence [32], and/or with genetic susceptibility (e.g. TNF-α polymorphisms) [33].

Although controversial, TNF-α polymorphisms were also correlated to metabolic syndrome and cholesterol levels [34]. In addition, TNF-α has multiple effects on adipocytes, including cell differentiation, lipolysis and production of adipokinin; moreover, TNF-α affects cholesterol efflux of adipocytes *in vitro* [35]. We can speculate that some types of TNF-α or other cytokine polymorphisms can predispose to a certain type of mucosal reaction to *H. pylori*, in parallel with a different lipidemic pattern. Nevertheless, investigation of the etiological relationship of this observation was beyond the aims of our study.

In conclusion, in dyspeptic patients, no difference in the lipid profile of *H. pylori*-infected individuals was found, with the exception of a tendency for CHOL levels to be lower. However, CHOL, HDL-C, LDL-C, apo-A and apo-B levels were all decreased in patients with uncomplicated DU, even though the CHOL/HDL-C ratio remained unchanged. The etiologic factor differentiating the lipid profile only in *H. pylori*-positive patients carrying a DU among dyspeptic patients could be dietetic, microbial, genetic or a combination of all three. Future studies evaluating the relationship between *H. pylori* and lipid profile must take into account the presence of DU as well as the identification of possible subgroups determined by the interaction of special genetic polymorphisms and *H. pylori* strains.

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Amino Acids (2008) 34: 413–420
DOI 10.1007/s00726-007-0554-8
Printed in The Netherlands

Amino Acids

Arabino-Galactan Proteins from *Pistacia lentiscus* var. *chia*: isolation, characterization and biological function

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Received March 1, 2007
Accepted April 3, 2007
Published online May 21, 2007;

Summary. Arabino-Galactan Proteins (AGPs) were isolated from Chios mastic gum (CMG) by using a buffer containing 0.1 M NaCl, 20 mM Tris–HCl, pH 7.5. Protein analytical methods, combined with specific procedures for carbohydrate characterization, indicated the presence of highly glycosylated protein backbone. In particular, staining by Yariv reagent of the electrophoretically separated molecules revealed the existence of arabinose and galactose and such a modification is characteristic for AGPs.

After experiments involving extensive dialysis of the isolated extracts against water and atomic absorption, there was evidence of the existence of zinc ions that are probably covalently bound to the AGPs. By using anion-exchange chromatography, capillary electrophoresis, colorimetric methods and GC-MS, it was found that the extracts were separated into three major populations (A, B, and C), which were consistent with their respective negative charge content namely, uronic acid. The characterization of neutral sugars that was investigated with GC-MS showed the existence of arabinose and galactose in different amounts for each group.

Experiments concerning the inhibition of growth of *Helicobacter pylori* in the presence of AGPs, as is shown for other CMG constituents, showed that the extracts of at least 1.4 g CMG affected the viability of the bacterium. There is no evidence as to whether the AGPs provoke abnormal morphologies of *H. pylori*, as is reported for the total CMG, or for O-glycans that possess terminal α1, 4-linked N-acetylglucosamine and are expressed in the human gastric mucosa; this has to be further investigated.

Keywords: Arabino-Galactan Proteins (AGPs) – Chios mastic gum (CMG) – Biological function

Introduction

Mastic is a white, semitransparent, natural resin that is obtained as trunk exudates from mastic trees. Its scientific name is *Pistacia lentiscus*, and it belongs to the Anacardiaceae family. The plant *Pistacia lentiscus* var. *chia*. grows particularly and almost exclusively in the south region of Chios Island, Greece, and produces a resin, known as Chios mastic gum (CMG). CMG, like other exudate gums, is produced from the trunk and the thickest branches of the tree when it is injured in order to seal the wound, preventing the infection and dehydration of the plant. The chemical composition of the mastic oil and the essential oil of the resin have been reported (Papageorgiou et al., 1991; Magiatis et al., 1999). Its biological activity can be attributed to a variety of compounds. It contains triterpenes of the oleanane, euphane and lupine type (Andrikopoulos et al., 2003; Assimopoulou and Papageorgiou, 2005), alpha-tocopherol (Kivcak and Akay, 2005) and polyphenols (Romani et al., 2002); the latter have been associated with a hypotensive effect of mastic (Sanz et al., 1992).

CMG possesses anti-bacterial properties (Iauk et al., 1996; Magiatis et al., 1999; Koutsoudaki et al., 2005) and its in vivo-demonstrated antiplaque action in the oral cavity has been attributed to its inhibitory action against overall bacterial growth (Takashi et al., 2003), and especially against *S. mutants* (Aksoy et al., 2006). As far as the effectiveness of *Pistacia lentiscus* against *Helicobacter pylori* and peptic ulcers is concerned, data remain controversial. Clinical studies have initially indicated that CMG is effective against gastric and duodenal ulcers (Al-Habbal et al., 1984; Al-Said et al., 1986); mastic has also been proven to be bactericidal against *H. pylori* in vitro (Huwez et al., 1998; Marone et al., 2001). However, other studies show that a total mastic extract without polymer led to an approximately 30-fold reduction in *H. pylori* colonization of infected mice (Paraschos et al., 2007), while the results of Loughlin et al. (2003) showed that *H. pylori* in infected

mice was not eradicated after CMG receipt. In addition, the inability of CMG to eradicate *H. pylori* from infected humans (Bebb et al., 2003) contradicted the findings reported by Al-Habbal et al. (1984) and Al-Said et al. (1986).

All the above activities concerning the therapeutic properties of CMG were explored by using either the total resin or the constituents that were extracted with buffers containing organic solvents. However, it is well known that exudate gums also contain substances that are water soluble and some of them are rich in arabinogalactan proteins (AGPs) (Showalter, 2001). AGPs are a family of extensively glycosylated hydroxyproline-rich glycoproteins, analogous to animal proteoglycans that are thought to have important roles in various aspects of plant growth and development. At the organ level, AGPs are found in leaves, stems, roots, floral parts and seeds (Fincher, et al., 1983; Nothnagel, 1997). Knowledge of the protein moieties of AGPs has mostly come from purifying AGPs, deglycosylating them and analyzing their respective core proteins by amino acid analysis and, to a more limited extent, by sequence analysis (Du et al., 1994; Gao et al., 1999). As their name implies, AGPs are rich in arabinose and galactose and in some cases glucuronic acid, along with other less abundant sugars. AGPs are thought to function in various aspects of plant growth and development namely, vegetative, reproductive and cellular growth and development (Cheung et al., 1995), programmed cell death and social control (Shindler et al., 1995) and molecular interactions and signaling (Schultz et al., 1998).

Recently it has been reported that AGPs from *Echinacea purpurea* stimulate phagocytosis and release of TNF by macrophages (Wagner et al., 1988; Classen et al., 2000), and a pilot study suggested that this AG might be effective in vivo in reducing chemotherapy-induced leucopenia (Melchart et al., 2002), as another AGP from *Echinacea purpurea* was found to possess complementary-stimulating activities in vitro (Alban et al., 2002), while interactions with leucocytes or the complementary system have been demonstrated by Diallo et al. (2001) and Thude et al. (2006), respectively.

Within this work we present the existence of AGPs in CMG extracts which are linked to zinc, as evidenced by atomic absorption. Their isolation as well as their partial characterization have shown that they consist of 3.1% protein, 78.5% neutral sugars (arabinose and galactose) and 18.4% uronic acids. In addition, it is evidenced by MIC experiments that they exhibit antibacterial activity in vitro against *H. pylori.*

Materials and methods

Preparation of water-soluble gum extracts and verification of their protein content

Dry Chios mastic gum (resin, CMG) was pulverized to a fine powder and 5 g were mixed with 0.1 M NaCl, 20 mM Tris–HCl, pH 7.5, and after overnight stirring at 4 °C were centrifuged to separate soluble (supernatant) material from insoluble (pellet) material. The soluble material was filtered by using 0.45 μm HA filters and dialyzed extensively against water. The zinc content of the extracts was estimated by atomic absorption on a Perkin-Elmer atomic absorption spectrometer (Model 403) at 214.5 nm and by using a Zn EDL lamp. The extracts were analysed on SDS-PAGE 12% and visualized with Coomassie blue. The presence of the protein was verified after incubation of the extracts with proteinase K. In particular, the extract from 2 g CMG was solubilized in 50 μl 100 mM Tris–HCl, pH 7.5, 0.5% SDS. Freshly prepared proteinase K stock solution (10 mg/ml in water) was added to a final concentration of 20 μg/ml and the mixture was incubated at 37 °C for 4.5 h. The sample was then analyzed in 12% SDS-PAGE.

Polyclonal antibodies production

The isolated extracts from CMG that were prepared as described above were used for polyclonal antibodies production. A 3-month-old rabbit was injected subcutaneously with the extracts from 1 g mastic as described by Harlow and Lane (1998). The resulted rabbit serum was collected after the second and third injection and was stored in 1ml aliquots at −20°C. The isolated CMG extracts were separated onto SDS-PAGE 12%, transferred onto PVDF membranes and visualized by using the produced antiserum in a dilution of 1:1000.

Glycosylation investigation of the CMG extracts

In order to investigate the modification of the proteins within the CMG extracts, a procedure was followed that concerns the glycoprotein oxidation, as described by Rothfus and Smith (1963). The protein extract (from 1 g CMG) was solubilized in 250 μl of 0.1 M Na-acetate buffer, pH 4.5 and was pre-cooled on ice. A fresh stock of 0.5 M $NaIO_4$ pre-cooled on ice and stored in the dark was prepared and was added to the sample to a final concentration of 10 mM periodate. The mixture was then incubated in the dark for 1 h at room temperature, the reaction was stopped by adding 1/10 volume of 0.5 M ethylene glycol and the excess of the glycol reagent was removed by dialysis against PBS.

The biotinylation of oxidized glycoproteins with the commercially available biocytin hydrazide (Pierce Chemical) was performed according to Spector et al. (1998). A freshly prepared stock solution of biocytin hydrazide (1 mg/ml in DMSO) was added to the dialyzed sample at a ratio of 1:10. This was followed by incubation of the samples at room temperature for 1 h, and the resulting biotinylated glycoproteins were analyzed on 12% SDS-PAGE and transferred on a nitrocellulose membrane, and the biotin was detected with the streptavidin-horseradish peroxidase protein.

Characterization of the CMG polysaccharide content

Extraction and purification of polysaccharides

CMG was extracted for 24 h at 4 °C under continuous stirring with 20 mM Tris/HCl, pH 7.5 which contained 0.1 M NaCl. After the end of extraction, the extract was filtered and centrifuged in order to remove the solid particles. Salts and other molecules of low molecular weight were removed from the extract with dialysis against water and gel filtration chromatography on PD-10 columns (Sephadex G-25, Pharmacia). Separation and isolation of carbohydrates was performed using an FPLC (Pharmacia) system with anion-exchange chromatography on Mono Q (Pharmacia)

column with a flow-rate of 1.0 mL/min. Elution was performed with gradient from 0.0 to 0.3 M NaCl for 25 min and from 0.3 to 1.0 M NaCl for 20 min in 20 mM Tris/HCl buffer, pH 7.5.

Colorimetric methods

The characterization of the crude extract and the isolated fraction was performed with colorimetric methods. Total neutral sugars and uronic acid were determined with the anthrone method, using galactose as standard (Scott and Melvin, 1953; Laurentin and Edwards, 2003), and the borate-carbazole method of Bitter and Muir, using the glucuronic acid lactone as standard (Bitter and Muir, 1962), respectively, after correcting for the interference of each of these substances with the other reaction. Total protein was determined with the Bradford method (Bradford, 1976), using bovine serum albumin as standard, and sialic acid with the orcinal reagent, using N-acetyl-neuraminic acid as standard (Schauer, 1978).

Other analytical methods

Purity and homogeneity of polysaccharide fractions was obtained using capillary electrophoresis. Capillary electrophoresis was performed in an HP3DCE instrument from Agilent Technologies in uncoated silica-fused capillaries (50 μm i.d. × 54.6 cm effective length). Separation was performed in a 25 mM borate buffer, pH 10.0, containing 25 mM SDS at 20 kV (normal polarity), and detection was at 200 nm. SDS-PAGE was performed as described by Laemmli using a 10% polyacrylamide gel in a vertical slab gel electrophoresis apparatus (Bio-Rad Laboratories). Staining of the gel was achieved with a combination of polysaccharide oxidation and Alcian-Blue staining (Moller and Poulsen, 1995). Determination of neutral and amino sugars was performed with GC-MS as TMS derivatives by the UCSD GRTC Glycotechnology Core Resource. In brief, methanolic HCl was used to hydrolyze glycosidic bonds and concomitantly form the methyl glycoside derivative (methanolysis). Free hydroxyl groups are trimethylsilylated and the resulting volatile derivatives are fractionated by gas chromatography on a DB-1 column using a temperature gradient and detected by electron ionization mass spectrometry (Kakehi and Honda, 1989).

Verification of the arabino-galactan presence in CMG extracts

Samples of mastic extract (from 1 g of mastic) were dissolved in Laemmli buffer and analysed on a 12% SDS-PAGE. The gel was then immersed for 15 min in β-glucosyl Yariv reagent. The concentration of Yariv reagent was 0.15 mg/ml in 1% NaCl (Baldwin et al., 1993).

Bactericidal activity of the water extracts against Helicobacter pylori

H. pylori strains were grown on agar plates containing 10% horse serum in a microaerophilic atmosphere (generated by Campy-Gen, Oxoid, Basingstoke, U.K.) at 37 °C for 48 h. Bacteria were harvested in phosphate-buffered saline (pH 7.4), diluted to a concentration of 2×10^9cells/ml and 10 μl were plated on 6 cm plates plus 10 μl BHI (Brain heart infusion – growth medium) containing agar. The mastic gum water soluble extracts of 1, 2 and 3 g were dissolved in 50 μl PBS and added to the cultures.

Results and discussion

Preparation and partial characterization of the proteinaceous nature of the water soluble CMG extracts

The pulverized resin (5 g each time), as described under the experimental session, was mixed with a buffer containing 0.1 M NaCl, 20 mM Tris–HCl, pH 7.5 and after overnight stirring at 4 °C was centrifuged to separate the soluble (supernatant) from the insoluble (pellet) material. Here it has to be pointed out that the above-used buffer was found to be more successful than others (data not shown) and therefore its use was adopted. After the centrifugation step, filtering as well as extensive dialysis against water were very important steps in order to remove salts and everything else that was co-purified without being covalent bound to the extracted substances. The extracts were analyzed with SDS-PAGE 12% and visualized with Coomassie blue (Fig. 1a). It is clearly shown that several bands with different molecular masses, ranging from 14 to 150 kDa, were present and that some of them were highly diffused. This electrophoretical behaviour was thought to be attributed to post translational modifications and in this particular case to glycosylation. The presence of glycosylated molecules within the isolated extracts as well as their partial characterization are described in detail below in this section.

These extracts were used for the production of polyclonal antibodies. Figure 1b shows a Western blot analysis of CMG extracts which were separated by SDS-PAGE 12% and transferred onto PVDF membranes. Their visualization was performed by using the produced antiserum in a dilution of 1:1000. The protein character of the analyzed substances was verified with proteinase K. Thus, the extracts obtained from 2 g CMG were incubated with

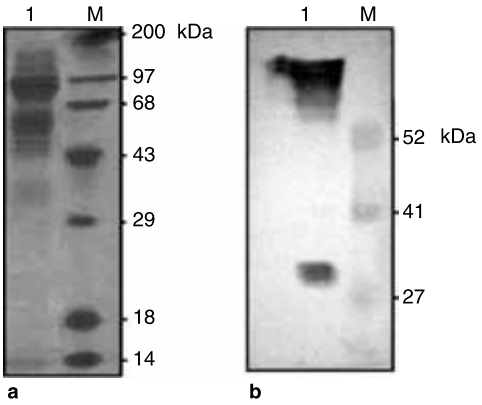

Fig. 1. Analysis of mastic gum extracts onto 12% SDS-PAGE. The extraction was performed with a buffer of 20 mM Tris–HCl pH 7.5, 100 mM NaCl, followed by extensive dialysis against water and drying in a Speedvac. **a** The existence of several bands indicates the presence of proteinacious molecules in the sample. **b** Western blot analysis of mastic gum extracts. Detection was performed with rabbit anti-mastic serum

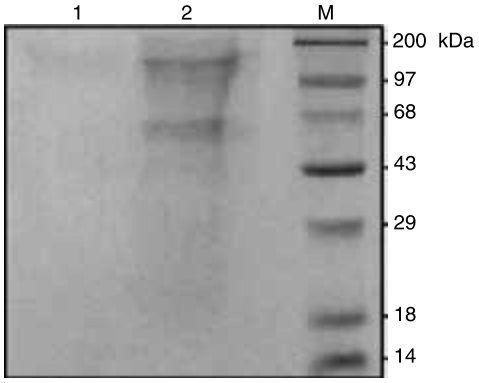

Fig. 2. Treatment of the extracts with Proteinase K. Mastic extracts were subjected to proteolysis with proteinase K at 37 °C for 4.5 h, analyzed onto 12% SDS-PAGE and visualized by staining with coommassie blue. *1* shows the sample after incubation with Proteinase K, *2*, the sample without protease treatment and *M* indicates the molecular weight markers

freshly prepared enzyme at 37 °C for 4.5 h and analyzed onto SDS-PAGE 12%. Figure 2 shows that when the extracts are incubated with proteinase K (lane 1) the bands disappeared, in contrast to the extracts that were not subjected to incubation with the enzyme (lane 2).

Another aspect that has also to be discussed here is the zinc assessment. The zinc content of CMG was reported several years ago. However, no evidence was reported until now for the co-existence of zinc within the different extracted substances. Figure 3 presents the zinc amount which was estimated with atomic absorption. CMG was subjected to extraction by using different buffers or solvents, as described in the legend of Fig. 3. Thus, it is

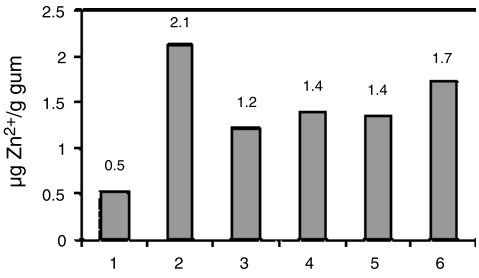

Fig. 3. Zinc assessments. The barograms show the zinc content after treatment of the extracts with different buffers/solvents and estimation with atomic absorption. *1* CMG extract with H_2O; *2* CMG extract with 0.1 M NaCl, 20 mM Tris–HCl pH 7.5; *3* CMG extract with HCl; *4* CMG extract with 0.5N HNO_3; *5* CMG extract with 0.1 M citrate-phosphate buffer; *6* CMG extracts with 6 M guanidine hydrochloride

clearly shown that the CMG extracts obtained by using the buffer containing 20 mM Tris–HCl, pH 7.5, 0.1 M NaCl exhibit the highest Zn concentration (Fig. 3, bar 2). It should also be noticed that the above assessments took place after extensive dialysis of the extracts against water by using the Spectrapor 3 dialysis membranes that allow molecules with MW lower than 3000 to penetrate them and therefore to be removed. The detection of Zn even under these conditions is indicated in Fig. 3. Its existence could probably be attributed to its specific or non-specific strong or covalent interactions with some parts of the isolated substances that inhibit its removal during dialysis.

Investigation of the carbohydrate moiety of the isolated substances

It has been mentioned above that some protein bands, which were visualized with Coomassie blue, were extremely diffused and this behaviour can be attributed to modifications such as glycosylation. In a first attempt, in order to characterize the modification, a $NaIO_4$-biocytin hydrazide coupled reaction was performed, as described under the experimental session. This methodology allows carbohydrate oxidation to occur by the addition of the $NaIO_4$ reagent. The formed aldehydes react with the hydrazide group of the biocytin-hydrazide reagent, which results in the labeling of carbohydrate molecules with biotin.

After labeling, the produced sample was analyzed by SDS-PAGE 12% and transferred onto PVDF membranes, and the detection was performed by using the streptavidin-horseradish peroxidase. In Fig. 4a, lane 1 clearly shows the presence of the carboxydrates. Positive and negative controls were used namely, fetuin (lane 2), a protein that is highly glycosylated, and albumin (lane 3), which is known not to carry such a modification, respectively. Figure 4b shows the deglycosylation of CMG extracts after treatment with $NaBH_4$ according to Gemmil and Trimble (1999). Visualization of the bands was performed after Western blot analysis by using the polyclonal antibodies that were raised against CMG extracts.

Further characterization of the highly glycosylated CMG extracts

In order to characterize the carbohydrates of CMG, chromatographic methods were carried out, as described under the experimental session. Neutral carbohydrate, uronic acid, sialic acid and protein analysis were tested for, according to Scott and Melvin (1953), Bitter and Muir (1962), and Bradford (1976) and Schauer (1978), respectively,

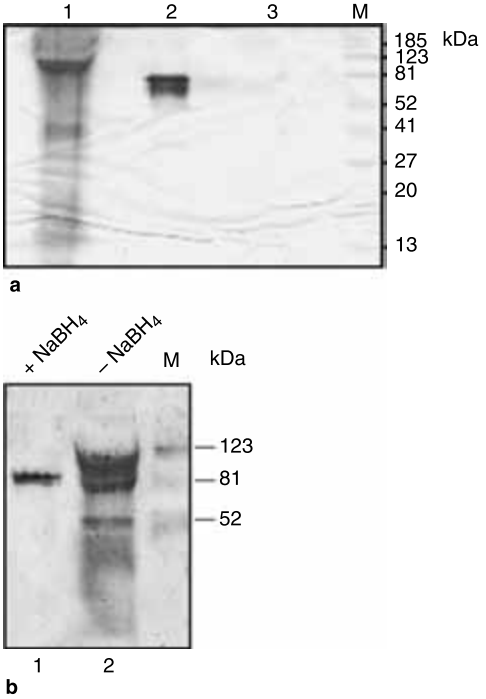

Fig. 4. Western blot analysis of the isolated proteins from GMG after biotinylation and treatment with $NaBH_4$. After treatment of the extracts with $NaIO_4$ for oxidizing the cis-1,2-diol groups of the carbohydrates and labeling with the biotin, the samples were analyzed onto SDS-PAGE 12%, transferred onto PVDF membranes and detected with the streptavidin-horseradish peroxidase. Positive reaction indicates the existence of carbohydrates in the sample. Deglycosylation with $NaBH_4$ was performed according to Gemmil and Trimble (1999). **a** *1* Glycosylation of the CMG extracts; *2* glycosylation of fetuin, which was used as positive control; *3* absence of glycosylation of albumin, which was used as negative control. *M* Molecular weight markers. **b** *1* CMG extracts after treatment with $NaBH_4$; *2* extracts without treatment

using galactose, glucurolactone, N-acetyl-D-neuraminic acid and bovine serum albumin as standards. The results of these experiments (Table 1) show that the major part of

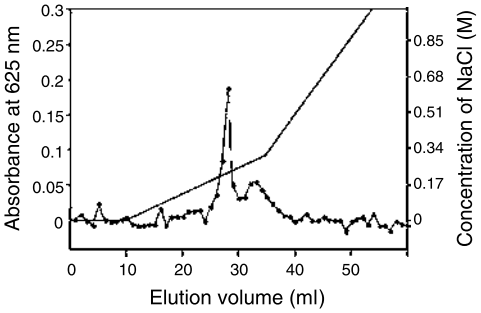

Fig. 5. Separation of the CMG extracts with ion-exchange chromatography. On this chromatogram there are 3 peaks dividing the fractions into three groups (Group A–C). Group A consists of the fractions eluted with 0.1–0.14 M NaCl and represents 7% of the eluted carbohydrates; group B consists of the fractions eluted with 0.18–0.23 M NaCl and represents 58% of the eluted carbohydrates; and group C consists of the fractions eluted with 0.26–0.37 M NaCl and represents 35% of the eluted carbohydrates

the extracts consists of neutral sugars (78.5%), uronic acids (18.4%) and proteins (3.1%), while there is a complete lack of sialic acids. The obtained CMG extracts were separated by using an ion-exchange MonoQ column, as shown in Fig. 5. On this chromatogram there are 3 peaks dividing the fractions in three groups namely, Group A, Group B and Group C. Group A consists of the fractions eluted with 0.1–0.14 M NaCl and represents 7% of the eluted carbohydrates; Group B consists of the fractions eluted with 0.18–0.23 M NaCl and represents 58% of the eluted carbohydrates; and Group C consists of the fractions eluted with 0.26–0.37 M NaCl and represents 35% of the eluted carbohydrates. As expected, the elution pattern of the three groups is consistent with their respective negative charge content namely, uronic acid. Chromatometric analysis of the three fraction groups is shown in Table 2. Thus Group A consists of 73.1% neutral sugars and 1.2% uronic acids, Group B consists of 50.5% neutral sugars and 2.54% uronic acids, while Group C consists of 49.3% neutral sugars and 3.3% uronic acids.

Table 1. Chromatometric analysis of CMG extract

	Concentration (μg/g of mastic)	Percentage
Proteins	21 ± 5	3.1
Carbohydrates	668	96.9
Neutral	541 ± 6	78.5
Uronic acids	127 ± 4	18.4
Sialic acids	–	–

Table 2. Carbohydrate analysis of fraction Groups A, B and C. All analyses were performed with chromatometric methods

	Group A (%)	Group B (%)	Group C (%)
Neutral sugars	73.1	50.5	49.3
Uronic acids	1.2	2.54	3.3
Proteins	Not detected	Not detected	Not detected

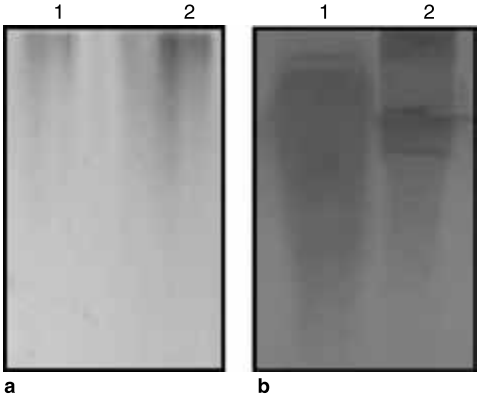

Fig. 6. Characterization of the carbohydrates with different staining procedures. **a** *1* Fraction group B, *2* fraction Group C. Both were visualized with Alcian blue. **b** Staining of the total CMG extracts with the Yariv reagent. *1* Arabic gum arabinogalactans (positive control), *2* CMG extracts

Proteins were not detected, probably due to their low amount and maybe due to the low sensitivity of the Bradford method. The existence of high molecular weight molecules in Groups B and C was additionally confirmed by Alcian blue staining after their separation onto SDS-PAGE 10% (Fig. 6a).

In order to elucidate their carbohydrate content, the macromolecules of groups B and C were subjected to GC-MS analysis. As shown in Table 3, the carbohydrates that were detected are arabinose and galactose. This is an indication that the glycosylated proteins of CMG belong to the family of AGPs, which is consistent with other proteinaceous macromolecules isolated from other gums (Verbeken et al., 2003). Experiments concerning the detection of CMG extracts by using the Yariv reagent, which is specific for AGPs detection, after their separation onto SDS-PAGE (Fig. 6b), have led to the same suggestions.

The homogeneity of Groups B and C was examined with capillary electrophoresis. Both B and C each form a single peak, migrating in 9.3 and 9.2 min, respectively

Table 3. Determination of neutral and amino sugars of fraction Groups B and C with GC-MS analysis

Neutral sugars	Group B (%)	Group C (%)
Arabinose	51.1	8.5
Galactose	48.9	91.5

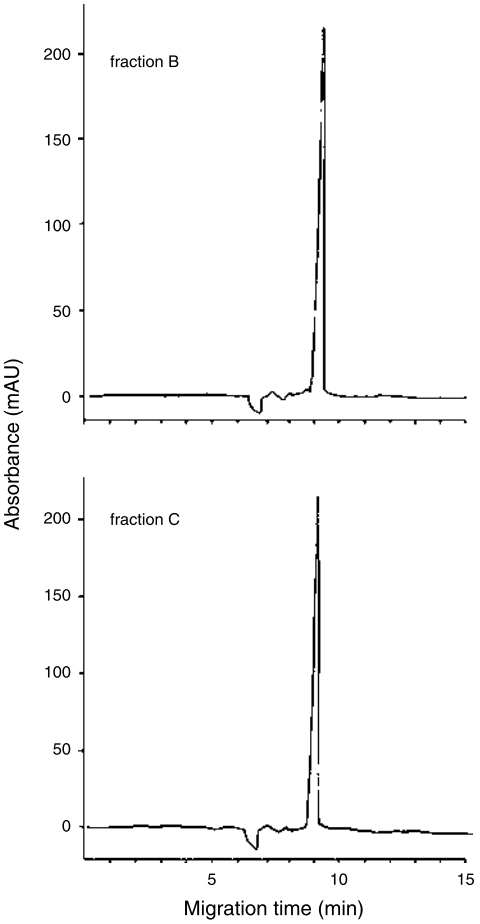

Fig. 7. Performance of purity and homogeneity of polysaccharide fractions by capillary electrophoresis. Capillary electrophoresis was performed in an HP3DCE instrument from Agilent Technologies in uncoated silica-fused capillaries (50 µm i.d. × 54.6 cm effective length). Separation was performed in a 25 mM borate buffer, pH 10.0, containing 25 mM SDS at 20 kV (normal polarity) and detection was at 200 nm. The sample from Group B migrates in 9.3 min and that from Group C migrates in 9.2 min, forming one peak each, indicating that the population in each sample is homogeneous

(Fig. 7), which verifies the homogeneity of the AGP populations.

Antibacterial activity

The bacteriostatic activity of AGPs against *H. pylori* was tested as described in the experimental session. According

Table 4. Growth of *H. pylori* in the presence of various CMG extracts

CMG extract	*H. pylori* growth
From 1 g CMG	+
From 1.4 g CMG	−
From 2 g CMG	−
From 3 g CMG	−
From 4 g CMG	−

to Table 4, the AGPs obtained from at least 1.4 g CMG inhibited the growth of the cells. However, such an effect of other AGPs has never been reported.

On the contrary, the antimicrobial activity of mastic against several pathogens was confirmed by several researchers, as mentioned in detail in the introduction. Although some of them reported that the inhibition efficiency of mastic against *H. pylori* in vitro and in vivo was effective in reducing *H. pylori* colonization and that the major triterpenic acids in the acid extract may be responsible for this activity (Paraschos et al., 2007), some others (Loughlin et al., 2003) showed that *H. pylori* in infected mice was not eradicated after CMG receipt. In addition, the inability of CMG to eradicate *H. pylori* from infected humans (Bebb et al., 2003) contradicted the findings reported by Al-Habbal et al. (1984) and Al-Said et al. (1986).

H. pylori colonizes the gastric mucosa by association solely with surface mucous cell-type mucin (Hidaka et al., 2001) and two carbohydrate structures, Lewis b and sialyl dimeric Lewis x in surface mucous cells that are serving as specific ligands for *H. pylori* adhesions BabA and SabA, respectively (Mahdavi et al., 2002). The pathogenic bacterium rarely colonizes the deeper portions of gastric mucosa, where the gland mucous cells producing mucins have terminal α1,4-linked N-acetyl-glucosamine residues attached to core 2-branched O-glycans (Nakayama et al., 1999).

Interestingly, Kawakubo et al. (2004) reported that these glycan chains that are produced by human gastric gland mucous cells raise the possibility of having protective properties against *H. pylori* infection. They found that this natural protection could be attributed to several abnormal morphologies of *H. pylori*, such as elongation, segmental narrowing and folding. The above-mentioned abnormal morphologies were observed by scanning electron micrographs of *H. pylori* after its incubation with the O-glycans that possess terminal α1, 4-linked N-acetylglucosamine.

In addition, Marone et al. (2001) evidenced in vitro the CMG total killing activity against *H. pylori,* attributing this effect to several morphological abnormalities, blebbing and cellular fragmentation that were caused by CMG.

By taking into account the observation reported by Marone et al. (2001) concerning the observed abnormal morphologies of *H. pylori* after its treatment with mastic, as well as the similar observations reported by Kawakubo et al. (2004), we suggest that the AGPs from CMG could provoke such alterations in *H. pylori* and therefore inhibit its growth in vitro. However, we cannot postulate that AGPs from CMG function in a similar way in vivo by altering the *H. pylori* morphology, as the mucin O-glycans protects the gastric mucous cells from infection. Their in vitro activity against *H. pylori* has to be further investigated in vivo.

Acknowledgments

We would like to thank the Chios Gum Mastic Growers Association for their financial support and the provision of mastic products. The authors would also like to express their thanks to Matthias Weber from Max-Planck Institute fuer Infektionsbiologie, Berlin for his technical assistance in *H. pylori* cultures and Mr Michalis Athanassiou for his technical assistance in the experiments concerning the carbohydrate analysis.

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Helicobacter pylori neutrophil-activating protein activates neutrophils by its C-terminal region even without dodecamer formation, which is a prerequisite for DNA protection – novel approaches against *Helicobacter pylori* inflammation

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Keywords
DNA binding; *Helicobacter pylori*; HP-NAP; neutrophil activation

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(Received 13 July 2007, revised 9 November 2007, accepted 20 November 2007)

doi:10.1111/j.1742-4658.2007.06201.x

Helicobacter pylori neutrophil-activating protein (HP-NAP) protects DNA from free radicals as a dodecamer through its ferroxidase activity without, however, directly binding to it. The retardation that was observed at pH 7.5 could be easily attributed to an iron effect, as it was revealed by experiments in the absence of HP-NAP. A total loss of ferroxidase activity, dodecamer formation and DNA protection in environments rich in free radicals was observed after replacement of His25, His37, Asp52 and Lys134, which are located within the ferroxidase site, with Ala. Molecular dynamics simulations revealed that dimer formation is highly unlikely following mutation of the above amino acids, as the Fe^{2+} is no longer attracted with equal strength by both subunits. These findings probably indicate that iron plays an important role in the conformation of HP-NAP by initiating the formation of stable dimers that are indispensable for the ensuing dodecamer structure. Very surprisingly, neutrophil activation appeared to be stimulated by structural elements that are localized within the C-terminal region of both mutant HP-NAP and wild-type dodecamer HP-NAP. In particular, the dodecamer conformation does not seem to be necessary for activation, and helices H3 (Leu69–Leu75) and H4 (Lys89–Leu114) or the linking coils (His63–Thr68 and Thr76–Ser88) are probably critical in stimulating neutrophil activation.

Helicobacter pylori neutrophil-activating protein (HP-NAP) is one of the virulence factors produced by the bacterium *H. pylori* [1]. This protein, originally purified from water extracts of *H. pylori*, was shown to induce neutrophil adhesion to endothelial cells *in vitro* [1] as well as *in vivo* [2], to increase the adhesion of neutrophils to endothelial cells [3], to induce migration and activation of human neutrophils and monocytes [4,5], and to be a potent stimulant of mast cells [6]. Its binding to neutrophil glycosphingolipids [7] and mucin, a component of the stomach mucous layer [8], has also been reported. HP-NAP-induced reactive

Abbreviations
AFM, atomic force microscopy; Dlp, Dps-like protein; Dpr, Dps-like peroxidase resistance; Dps, DNA-protecting protein; fMLP, formyl-Met-Leu-Phe peptide; HP-NAP, *Helicobacter pylori* neutrophil-activating protein; HP-NAPmut, mutant *Helicobacter pylori* neutrophil-activating protein; HP-NAPwt, wild-type *Helicobacter pylori* neutrophil-activating protein; LPS, lipopolysaccharide; MD, molecular dynamics; ROI, reactive oxygen intermediate; SOD, superoxide dismutase.

oxygen intermediate (ROI) production involves a cascade of intracellular activation events, including an increase in cytosolic Ca^{2+} concentration and phosphorylation of cytosolic proteins, leading to the assembly of the superoxide-forming NADPH oxidase on the neutrophil plasma membrane [5,9,10].

HP-NAP is a dodecameric protein consisting of 17 kDa monomers with a central cavity where iron ions bind [11,12]. The observation that its synthesis is not affected by the iron content of the growth medium led to the proposal that the primary role of HP-NAP *in vivo* may not be to scavenge iron [13].

The primary sequence and overall structure of HP-NAP [14] is similar to those of the DNA-protecting protein (Dps) family of iron-binding and DNA-protecting proteins [15].

Members of the Dps family protect DNA from oxidative damage through direct interaction. Dps and DNA form a highly ordered and stable nucleoprotein complex called a biocrystal, so that the DNA is 'sheltered' from the attack of the free oxidative radicals [16] produced by the Fenton reaction [17]. These proteins are present in many prokaryotes [18–23]. They bind ferrous ions, and some of them lack the ability to bind DNA *in vitro* [12,19,24].

The role of HP-NAP in protecting *H. pylori* from oxidative damage was first suggested by the observation that loss of alkyl hydroperoxide reductase leads to a concomitant increase in HP-NAP expression [25]. Like that of other Dps family members, HP-NAP production is maximal in stationary-phase cells, and an *H. pylori* napA mutant exhibits lower survival rates than the wild-type strain upon exposure to oxidative stress conditions [26]. Although results from *in vitro* DNA-binding assays suggest that the protein does not bind DNA [12], other data demonstrated that it binds DNA *in vitro* [27], or that it colocalizes with the nucleoid [26], suggesting that it may interact with DNA. According to Ceci *et al.* [28], HP-NAP adopts a mechanism different from that of *Escherichia coli* Dps to bind and condense DNA. This new information was obtained from gel retardation assays performed at different pH values and with atomic force microscopy (AFM). However, these results are not in accordance with those published by Wang *et al.* [29], who postulate that HP-NAP affects DNA mobility strongly at pH 8.0. The obtained retardation is similar to that reported by Ceci *et al.* [28] for pH 6.5 and pH 7.0, and not to that for pH 8.0. Studies by Ceci *et al.* [28] show that, at pH 8.0, the DNA retardation is minimal but the AFM imaging is similar to that observed at pH 7.5.

Concerning the involvement of HP-NAP in signal transduction events in eukaryotic cells, there are no published data concerning the probable involvement of other Dps family members, except for HP-NAP [10], and therefore its ability to induce a series of such events in eukaryotic cells makes HP-NAP distinct from other proteins of the Dps family.

In an attempt to further investigate the structure–function relationships of HP-NAP from *H. pylori*, focusing mostly on DNA binding, DNA protection, and neutrophil activation, the recombinant wild-type protein and its mutant form, obtained after replacing the crucial amino acids at the ferroxidase site, were overexpressed and purified. DNA shift assays under various conditions (pH, buffers) as well as ferroxidase activity experiments revealed that HP-NAP does not bind DNA, and therefore protection of DNA by means of ferroxidase activity occurs by a mechanism similar to that suggested for other non-DNA-binding Dps family members.

A possible mechanism of dimer formation was also investigated by molecular dynamics (MD) simulation. It seems that the ferroxidase site amino acids are indispensable for dimer formation, and that ferrous ions contribute extensively to the stability of the dimers in solution.

Concerning the neutrophil activation, it was found that the C-terminal region (HP-$NAP_{58–144}$) is probably critical in stimulating neutrophils. This region includes helices H3 (L69–L75) and H4 (K89–E114) and the linking coils (His63–Thr68 and Thr76–Lys83) that are apparently exposed in both the dodecameric and monomeric forms.

These findings provide a deeper understanding of the multiple functions of HP-NAP in protecting bacterial DNA, preventing the adverse effects of Fenton chemistry, and thereby providing a molecular explanation for the conservation of its characteristic intersubunit ferroxidase site. Our findings also provide an explanation for the activity of HP-NAP in production of ROIs following interaction with human leukocytes, thus suggesting new approaches for the development of therapeutic drugs, using peptide sequences as scaffolds for the rational design of new inhibitory molecules.

Results

Expression and purification of wild-type HP-NAP (HP-NAPwt), mutant HP-NAP (HP-NAPmut), HP-$NAP_{1–57}$ and HP-$NAP_{58–144}$ regions and dodecamer investigation

Genomic templates of HP-NAPwt, HP-NAPmut and its N-terminal and C-terminal regions were amplified

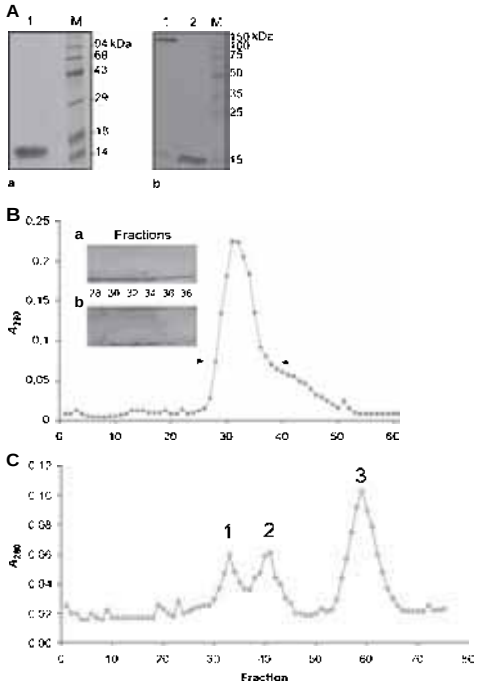

Fig. 1. Purification and dodecamer formation of recombinant HP-NAPwt by using Sephacryl S-200 gel chromatography and 12% SDS/PAGE. (A) Purified HP-NAPwt [(a) lane 1, and (b) lane 2] migrates at approximately 15 kDa. The protein band that migrates at 150 kDa [(b) lane 1] corresponds to HP-NAPwt after subjection to electrophoresis without prior boiling and in the absence of reducing reagents such as β-mercaptoethanol. The band that appears at 15 kDa [(b) lane 2] corresponds to the same protein after boiling and in the presence of β-mercaptoethanol. (B) Sephacryl S-200 gel chromatography of HP-NAPwt. The buffer was 20 mM phosphate (pH 7.5) and 150 mV NaCl, and the flow rate was 0.125 mL·min^{-1}. The volume of each collected fraction was 4 mL. The arrows show the analyzed fractions on 12% SDS/PAGE. (a) With mercaptoethanol and boiling. (b) In the absence of mercaptoethanol and boiling. (C) Sephacryl S-200 gel chromatography of markers with known molecular masses, using the same conditions as above. Peak 1 corresponds to aldolase (160 kDa), peak 2 to albumin (68 kDa) and peak 3 to cytochrome *c* (14 kDa).

by PCR, and the respective proteins were purified as described in Experimental procedures. The purification of HP-NAPwt cloned in the vector pET11a was carried out by ammonium sulfate precipitation, followed by anion exchange column chromatography (DEAE–sepharose) to remove traces of DNA non-specifically bound to the protein as detected by 1% agarose electrophoresis (data not shown). Fifteen milligrams of highly purified HP-NAPwt was isolated from a 1 L culture (Fig. 1Aa).

Furthermore, the protein eluate was also passed through Sephadex G-200, and its dodecameric conformation was ascertained after correlation of the elution volume with that of protein markers with known molecular masses (Fig. 1B,C). The ability of HP-NAPwt to form dodecamers was additionally verified by using 12% SDS/PAGE without prior boiling of the samples and in the absence of reducing reagents such as β-mercaptoethanol (Fig. 1Ab,B). This technique was established for SH-group-containing proteins. After analysis of the fractions that are marked by arrows in Fig. 1B without β-mercaptoethanol and boiling (Fig. 1Bb), two protein bands appeared. In contrast, the same fractions gave only one band following classic SDS/PAGE analysis, namely addition of β-mercaptoethanol and boiling (Fig. 1Ba). The SDS concentration for the separating and stacking gel was 0.5% w/v, and for the sample buffer it was 2% w/v.

pET11a HP-NAPmut was not easily purified, like the wild-type, and some other 'theoretically nonpermissible' modifications were included in the purification protocol, such as its passage through Ni–nitrilotriacetic acid affinity beads, which are normally used for His-tagged molecules (Fig. 2Aa, lane 1). The protein was bound onto the 'His-affinity' beads, probably by means of its iron ion affinity, and purified to a high degree. An anion exchange DEAE–sepharose column purification step or Sephadex G-200 were not necessary, because the protein was not contaminated by traces of DNA or RNA (data not shown). Its inability to form dodecamers was shown by SDS/PAGE (Fig. 2Aa, lane 2). HP-NAPwt tagged with 6× His was purified by using the protocol for Ni–nitrilotriacetic acid beads (Fig. 2Ab, lane 2, and Fig. 2Ba, lane 1). Its ability to form dodecamers is shown in Fig. 2Ab (lane 1).

The N-terminal and C-terminal fragments of HP-NAP were purified by affinity chromatography using Ni–nitrilotriacetic acid beads in the presence of 6 M urea and elution with the same binding buffer, including a high imidazole concentration (300 mM) (Fig. 2Ba, lane 2, and Fig. 2Bb, lane 1, for HP-NAP$_{58–144}$ and HP-NAP$_{1–57}$, respectively). The entire proteins, as well as their fragments, were treated with magnetic beads for lipopolysaccharide (LPS) removal as described under Experimental procedures.

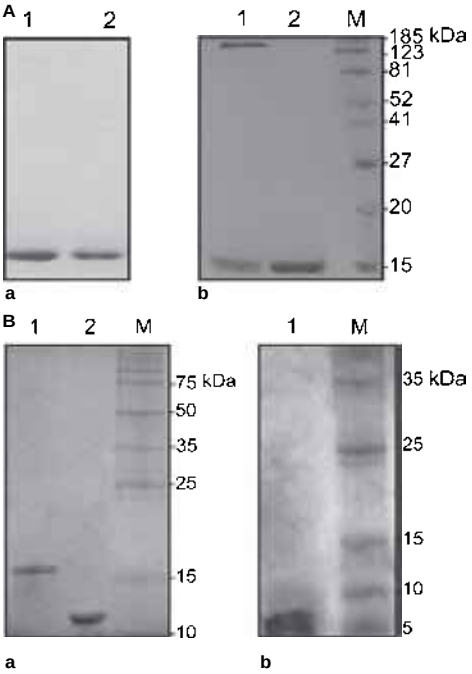

Fig. 2. Electrophoresis of HP-NAPmut, and His-tagged HP-NAPwt, HP-$NAP_{58–144}$ and HP-$NAP_{1–57}$, on SDS/PAGE. (A) (a) Lane 1 and lane 2 show recombinant HP-NAPmut with or without reducing agents and boiling. (b) Lane 1 and lane 2 show His-tagged HP-NAP-wt without or with boiling and reducing agents, respectively. SDS/PAGE was 12% for both cases. (B) (a) Lane 1 and lane 2 show His-tagged HP-NAPwt and HP-$NAP_{58–144}$ (over 10 kDa), respectively (SDS/PAGE, 15%). The His-tagged HP-$NAP_{1–57}$ is shown in (Bb), lane 1, at approximately 5 kDa (SDS/PAGE, 20%).

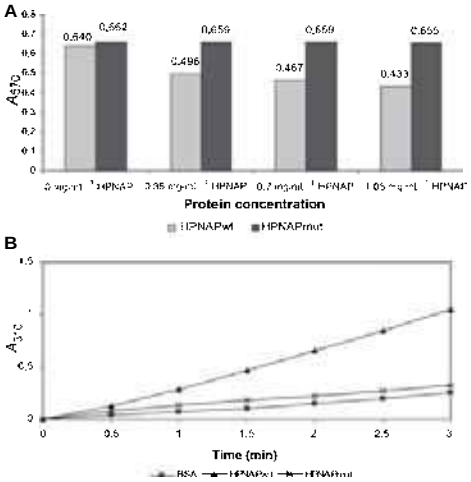

Fig. 3. Ferroxidase activity of HP-NAPwt and HP-NAPmut. (A). Increase of HP-NAPwt concentration in the reaction mixture led to a decrease in the remaining Fe^{2+}, showing the ferroxidase activity of the protein. On the other hand, increased concentrations of HP-NAPmut had no effect on the concentration of Fe^{2+}. (B) Time course of Fe^{2+} by HP-NAPwt (▲), HP-NAPmut (×) and BSA (■), 20 $\mu g \cdot mL^{-1}$, respectively. Data points are the means of three independent experiments.

Iron incorporation and ferroxidase activity

The ferroxidase activity of HP-NAPwt and HP-NAPmut is shown in Fig. 3A (gray and black bars, respectively). The mutated protein loses its ability to take up iron, due to the absence of the dodecamer structure (black bars). Figure 3B shows the iron uptake of both HP-NAPwt and HP-NAPmut.

MD simulations and dodecameric assembly

The association of HP-NAP monomers to form dodecamers can proceed in many ways, including formation of dimers or trimers, subsequent association of dimers, and so on. The first and most crucial step is the formation of a stable dimer in an up–down configuration (Fig. 4A). In the absence of ferrous ions, the types of residues that make up the interface between two monomers suggest that hydrophobic interactions make a large contribution to the stability of the dimer, and that hydrogen bonding is also involved in stability. However, the presence of ferrous ions at the active site is mainly responsible for dimer stability, as made clear by the analysis in supplementary Doc. S1.

The equilibrated structures of the HP-NAP monomers in the dimers AD-wt and AD-4mut do not show large backbone differences (rmsd = 1.222), although the structural changes caused by the mutations lead to a less stable dimer, as shown in the analysis in supplementary Doc. S1.

The number of hydrogen bonds connecting the monomers in the dimer is four in the wild-type and only two in the mutant (Tables 1 and 2). In the wild-type, two Fe^{2+} are 'coordinated' between the two monomers A and D via electrostatic bridges (Table 3, Fig. 4A), contributing to the stability of the dimer. In the absence of Fe^{2+}, the charges of A-Asp52, A-Glu56 and D-His25 and their symmetric D-Asp52, D-Glu56

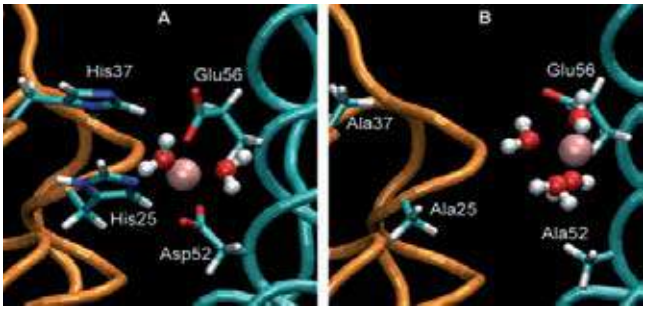

Fig. 4. Ferroxidase site of HP-NAP. (A) The 'ferroxidase site' in the equilibrated wild-type. The Fe^{2+} (pink) is kept in position by Asp52, Glu56, His25, and His37. Two water molecules are attracted by Fe^{2+}. (B) The same site in the equilibrated mutant. The Fe^{2+} is attracted one-sidedly by Glu56 and Asp53 (not shown), losing its ability to stabilize the dimer. Four water molecules are attracted by Fe^{2+}.

and A-His25 would hinder the approach of the two monomers to each other. In the mutant, the substitution of D-His25 by D-Ala25 and of A-Asp52 by A-Ala52 causes a shift of Fe^{2+} in the equilibrated structure so that it approaches that of A-Glu56 and A-Asp53 (Fig. 4B), thereby destabilizing the contacts between the monomers. On the other hand, A-Ala52 may contribute to the stability via hydrogen bonding to D-Trp26 and hydrophobic interactions. The positions of A-Ala37 and A-Ala25 do not allow them to approach chain D removing water molecules, and, therefore, they cannot contribute to the stabilization of the dimer. After reaching equilibrium in AD-wt, two water molecules solvate the Fe^{2+}, which is bridged to A-Asp52, A-Glu56, and D-His25 (Fig. 4A). In the case of AD-4mut, four water molecules solvate the Fe^{2+}, which is bridged to Glu56 and Asp53 (Fig. 4B), thus resembling a hexahedral geometry. In both cases, the Fe^{2+} is not fully hydrated and never leaves the protein in the course of the simulation. On the other hand, in AD-5mut, as expected, Fe^{2+} leaves its position in the hydrophobic pocket and migrates in three steps to a new stable position (after $\sim$ 420 ps) about 7 Å away, 'coordinated' perfectly in a hexahedral manner by the exact same six water molecules.

Table 1. Hydrogen bonds between monomers A and D in the equilibrated wild-type.

Chain	Residue	Group	Chain	Residue	Group
A	Tyr44	OH	D	Asp52	OD2
A	Ser70	N	D	Glu80	OE1
D	Tyr44	OH	A	Asp52	OD1
D	Leu69	N	A	Glu80	OE2

Table 2. Hydrogen bonds between monomers A and D in the equilibrated mutant.

Chain	Residue	Group	Chain	Residue	Group
D	Thr84	OG1	A	His64	ND1
D	Trp26	NE1	A	Ala52	O

Table 3. Bridges between Fe^{2+} and negatively charged groups of monomers A and D in the wild-type HP-NAP dimer.

Chain	Residue	Group	Chain	Residue	Group
A	Asp52	OD2	D	His25	NE2
A	Glu56	OE2			
A	His25	NE2	D	Asp52	OD2
			D	Glu56	OE2

In order to determine the effect of the mutations on the stability of the dimer, the ratio $K^{\mathrm{mut}}/K^{\mathrm{wt}}$ was calculated of the dimerization equilibrium constants for the mutant and the wild-type in the presence of bound Fe^{2+} (see supplementary Doc. S1).

$$\frac{K^{mut}}{K^{wt}} = \frac{e^{-\Delta F^{mut}/RT}}{e^{-\Delta F^{wt}/RT}} \approx 0$$

where ΔF is the Helmholtz free energy for the dimerization reaction.

We notice that the largest contribution to the difference between the free energies arises from the difference between the interaction energies of the Fe^{2+} with its environment in the wild-type and in the mutant. According to this, the ferrous ions make the wild-type dimer much more stable than the mutated one.

DNA-binding capacity determined by gel retardation assays and DNA protection against hydroxyl radicals

The DNA-binding capacity of HP-NAP was assayed under several conditions, as described in Experimental

procedures. pTZ-S14 recombinant plasmid and HP-NAP loaded with iron (0.5 mM) were incubated in the presence of 20 mM phosphate buffer and 50 mM NaCl (pH 6.5), or 20 mM Hepes and 50 mM NaCl (pH 7.5), for 30 min at 37 °C. In addition, the plasmid was incubated with the same amount of protein for different time periods, namely 60, 90 and 120 min, at 4 °C by using 20 mM phosphate buffer and 50 mM NaCl at pH 6.5. The DNA mobility was investigated with 1% agarose gel as shown in Fig. 5A–C. Figure 5A (lane 2) shows the effect of iron without the protein, and lanes 1 and 3 indicate the DNA or the incubation mixture DNA and HP-NAP/DNA, respectively. The buffer was 20 mM Hepes and 50 mM NaCl (pH 7.5), and the incubation conditions were 30 min and 37 °C. It is clearly shown that the DNA at pH 7.5 was retarded even after iron incubation without HP-NAP, which points to an effect of iron itself. Figure 5B presents the same experiment using different buffers, namely 20 mM phosphate and 50 mM NaCl (pH 6.5), keeping all other conditions constant. Thus, iron-incubated DNA (lane 1) was not retarded, the faster-migrating band of DNA (lane 3) almost disappeared, and the bands with a lesser degree of supercoiling were stronger. The addition of HP-NAP (lane 2) stabilized the DNA band with the lesser degree of supercoiling, but did not induce retardation. Figure 5C shows the kinetics of the reaction. The buffer was 20 mM phosphate and 50 mM NaCl (pH 6.5), and the incubation time ranged from 60 min to 120 min at 4 °C. Lane 1 shows the plasmid DNA, and lane 2 (iron and DNA) and lane 3 (DNA and HP-NAP) correspond to mixtures incubated for 60 min. Lane 4 (iron and DNA) and lane 5 (DNA and HP-NAP) correspond to 90 min, and lane 6 (iron and DNA) and lane 7 (DNA and HP-NAP) correspond to 120 min. The upper DNA band with the lesser degree of supercoiling seems to be the dominant form at all time periods used, and the retardation appeared to be induced by using Hepes pH 7.5, even without incubation with the protein. From the above observations, we cannot postulate that the retardation is caused by the formation of a complex between the plasmid DNA and the protein. This is in agreement with the results of Tonello *et al.* [12], but different from those of Bijlsma *et al.* [27], Cooksley *et al.* [26], Ceci *et al.* [28], and Wang *et al.* [29]. As mentioned briefly above, the results of Ceci *et al.* [28] and Wang *et al.* [29] are not in agreement, because they present different degrees of retardation at different pH values. Our results are discussed in detail in the Discussion.

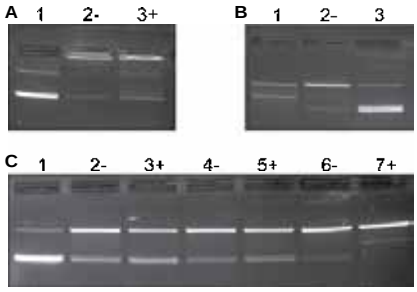

Fig. 5. Gel retardation assays of HP-NAPwt and DNA. The faster-migrating bands correspond to the plasmid with the highest degree of supercoiling; the slower-migrating bands correspond to a lesser degree of supercoiling and to the circular plasmid. (A) Lane 1: plasmid DNA pTZ-S14 *TthS14* gene. Lane 2: DNA incubated at 37 °C for 30 min with 0.5 mM Fe^{2+}. Lane 3: DNA incubated with Fe^{2+}-loaded HP-NAP, under the same conditions. The buffer used was 20 mM Hepes and 50 mM NaCl (pH 7.5). (B) Lane 1: plasmid DNA incubated with 0.5 mM Fe^{2+}. Lane 2: DNA incubated with Fe^{2+}-loaded HP-NAP. Lane 3: plasmid DNA. The incubation conditions were as above, and the buffer used was 20 mM phosphate and 50 mM NaCl (pH 6.5). (C) Lane 1: plasmid DNA. Lane 2: DNA incubated with 0.5 mM Fe^{2+} for 60 min. Lane 3: DNA incubated for 60 min with Fe^{2+}-loaded HP-NAP. Lanes 4 and 5 show DNA incubated with 0.5 mM Fe^{2+} and DNA incubated with Fe^{2+}-loaded HP-NAP for 90 min, respectively. Lanes 6 and 7 show DNA incubated with 0.5 mM Fe^{2+} and DNA incubated with Fe^{2+}-loaded HP-NAP for 120 min, respectively. The buffer in all these cases was 20 mM phosphate and 50 mM NaCl (pH 6.5), and the incubation temperature was 4 °C.

Martinez & Kolter [30] suggested that Dps family members afford protection of DNA from cleavage by radicals produced in Fe^{2+}-mediated Fenton reactions. This protection is due to a physical association between the two macromolecules. On the other hand, a member of the Dps family, from *Agrobacterium tumefaciens*, was shown to protect DNA from radicals without complex formation with DNA [19].

In an attempt to further elucidate the ability of the protein to protect DNA from oxidative stress, an *in vitro* DNA damage assay was set up. The *TthS14* gene (183 bp) was incubated with a solution containing 0.5 mM $Fe(NH_4)_2SO_4$, in the presence or absence of recombinant HP-NAP generated from pET11a constructs, for different incubation periods (from 15 min to 1 h). Figure 6 (lanes 2, 4, 6 and 8) shows the DNA protection in the presence of HP-NAPwt for 15, 30, 45 and 60 min, respectively. Figure 6 (lanes 10 and 11) shows the DNA degradation in the absence and presence of HP-NAP for 15 min, respectively. These findings are in accordance with the behavior of Dps from

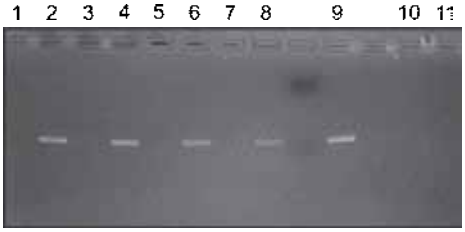

Fig. 6. DNA protection experiments on HP-NAPwt and HP-NAPmut using the *TthS14* gene, analyzed with 1% agarose gels. Lane 1: DNA exposed to 0.5 mM Fe^{2+} for 15 min. Lane 2: DNA with HP-NAPwt, exposed to Fe^{2+} for 15 min. Lane 3: DNA exposed to 0.5 mM Fe^{2+} for 30 min. Lane 4: DNA with HP-NAPwt, exposed to Fe^{2+} for 30 min. Lane 5: DNA exposed to 0.5 mM Fe^{2+} for 45 min. Lane 6: DNA with HP-NAPwt, exposed to Fe^{2+} for 45 min. Lane 7: DNA exposed to 0.5 mM Fe^{2+} for 60 min. Lane 8: DNA with HP-NAPwt, exposed to Fe^{2+} for 60 min. Lane 9: nonexposed DNA. Lane 10: DNA exposed to 0.5 mM Fe^{2+} for 15 min. Lane 11: DNA with HP-NAPmut, exposed to Fe^{2+} for 15 min.

A. tumefaciens, which does not bind DNA but protects it from Fenton reaction products [19].

Neutrophil binding and activation

HP-NAP, as a member of the Dps family, has the ability to protect *H. pylori* from oxidative stress. This was shown by the observation that loss of alkyl hydroperoxide reductase leads to a concomitant increase in HP-NAP expression [25]. These properties, as well as its ability to stimulate the production of ROIs by human neutrophils and monocytes, are associated with the structure–function relationships of the protein [5,11].

In order to further investigate neutrophil activation, human neutrophils were isolated from healthy donors, and their activation was measured in terms of assessment of the amount of superoxide anions produced via the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c* assay, as described in detail in Experimental procedures.

A closer look at the structure of the dodecamer revealed that helices H3 and H4 containing the sequences LSEAIKL(69–75) and SKDIFKEILEDYKYLEKEFKELSNTA(88–113), respectively, as well as the linking coils His63–Thr68 and Thr76–Lys83, are localized on the surface of the dodecameric structure, and these were chosen as possible candidates for neutrophil binding and activation (Fig. 7A,B). According to the above suggestion, the N-terminal region should not bind to neutrophils, whereas the C-terminal region would account for neutrophil activation.

To investigate the role of these regions, a new set of constructs containing the entire protein (wild-type and mutant) as well as HP-$NAP_{1–57}$ and HP-$NAP_{58–144}$ were cloned into the pET29c expression vector and purified as described in Experimental procedures. All entire proteins used (HP-NAPwt, HP-NAPmut), as well as the N-terminal and C-terminal fragments, were treated with polymixin B-coated beads for LPS removal prior to neutrophil activation. The results, which are shown in Fig. 8A, show the activation of neutrophils by both HP-NAPwt (0.234) and HP-NAPmut (0.214). The absorptions obtained prior to LPS removal were 0.250 and 0.220, respectively. These findings show that binding to neutrophil receptors can probably be attributed to protein elements that are exposed and are localized on the surface of the protein, and not solely to the dodecamer conformation itself. Figure 8B indicates that neutrophils are activated by the entire protein as well as by HP-$NAP_{58–144}$, with absorptions of 0.234 and 0.201, respectively, assessed at 550 nm. Their observed absorptions prior to LPS removal were 0.250 and 0.210, respectively. Concerning HP-$NAP_{1–57}$, the absorption obtained before LPS removal was 0.110, and that after the treatment 0.106, as shown in Fig. 8B.

Experiments were also performed at the same time with the same neutrophil preparation, by using a 6× His peptide that was synthesized in order to investigate possible neutrophil activation resulting from the constructs' His tags (Fig. 8B). Indeed, the results showed that when the absorption of hexapeptide (0.082) was subtracted from that of HP-NAPwt (Fig. 8C) and HP-$NAP_{58–144}$ (Fig. 8), the remaining values were 0.152 and 0.119, respectively. In contrast, the remaining absorption concerning HP-$NAP_{1–57}$ was reduced to 0.024 units (Fig. 8C).

Discussion

This article is concerned with the structure–function relationships of HP-NAP at several levels. Its ability to protect DNA from free radicals as a dodecamer through its ferroxidase activity without directly binding to it was investigated as described in Results. The recombinant protein produced from the pET11a plasmid was easily purified, and its dodecamer formation was shown by gel exclusion chromatography on Sephacryl-S200. The eluted fractions from the column that contained the protein were analyzed by SDS/PAGE in the absence or presence of reducing agents such as β-mercaptoethanol and boiling (Fig. 1Ba,Bb). The method was developed for cysteine-containing proteins. However, very surprisingly, the

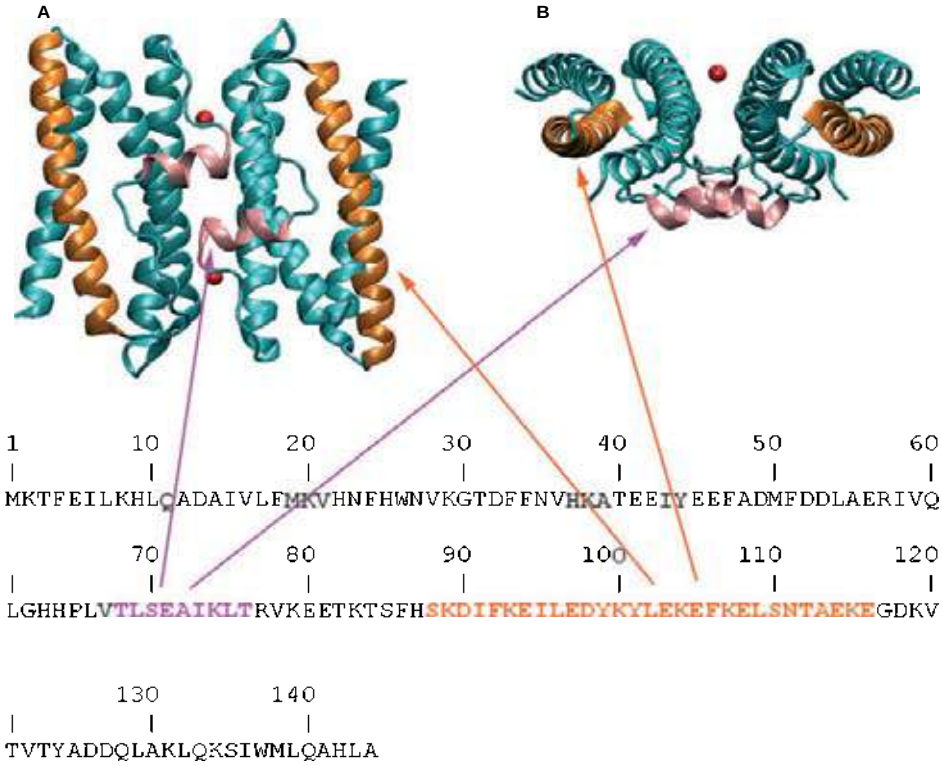

Fig. 7. Schematic representation of exposed helices of HP-NAP HP-NAP dimer in stand up view (A) and top view (B), with the exposed helices H3 and H4 (therefore suitable candidates for interacting with the neutrophils) colored in violet and orange respectively.

formation of higher-order conformations, even for HP-NAP that does contain cysteine residues, was seen when β-mercaptoethanol and boiling were avoided.

In an attempt to further elucidate the ability of the protein to form dodecamers by using SDS/PAGE, purified HP-NAP was analyzed by avoiding only the boiling of the sample prior to electrophoresis (data not shown) in the presence of β-mercaptoethanol. Indeed, the formation of higher-order conformations was again seen. It seems that heating disrupts the interactions between the monomers.

Additionally, the dodecamer formation of His-tagged HP-NAPwt was also investigated by SDS/PAGE, as shown in Fig. 2Ab. In contrast, recombinant HP-NAPmut (His37, Asp52, and Lys134, which are located within the ferroxidase site, were replaced by Ala) produced from the pET11a plasmid could not form dodecamers, as shown in Fig. 2Aa (both lanes). These results are in accordance with our theoretical results obtained with MD simulations. MD simulations revealed that dimer formation is highly unlikely following mutation of the above amino acids, as the Fe^{2+} is not attracted equally strongly by both subunits. These findings indicate that iron plays an important role in the conformation of HP-NAP by initiating the formation of stable dimers that are indispensable for the ensuing dodecamer structure.

Concerning DNA interaction and protection, several studies have been published with controversial results: namely, Tonello *et al.* [12] referred to the inability of

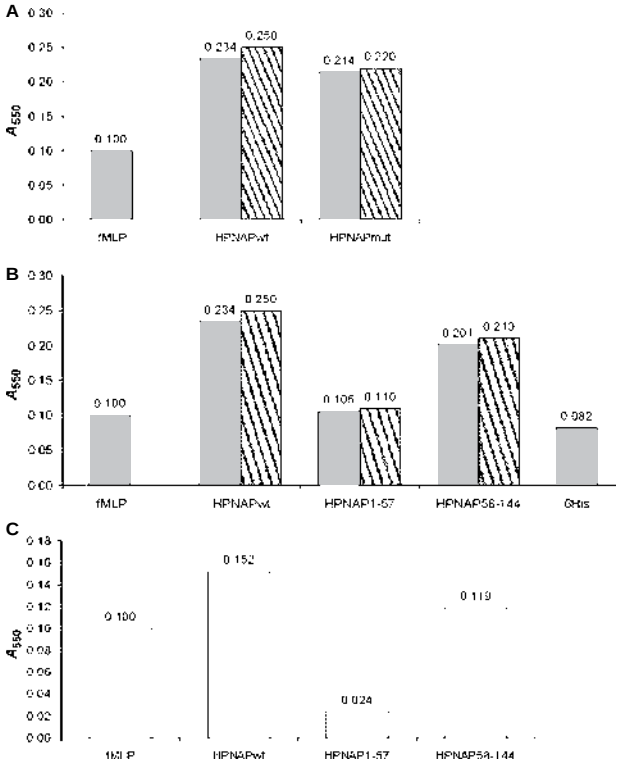

Fig. 8. Neutrophil activation measured at $A_{550\ nm}$. ■, Activation after treatment with polymixin B-coated magnetic beads for LPS removal. ■, Activation before treatment. □, Activation after subtraction of the 6× His value from those of HP-NAPwt, HP-$NAP_{1–57}$ and HP-$NAP_{58–144}$. fMLP peptide was used as control for all cases, and the protein concentration for all cases was 1 μM. (A) Neutrophil activation by HP-NAPwt after and before treatment (0.234 and 0.250, respectively) and by HP-NAPmut after and before treatment (0.214 and 0.220, respectively). (B) Neutrophil activation by HP-NAPwt after and before treatment (0.234 and 0.250, respectively), by HP-$NAP_{1–57}$ after and before treatment (0.106 and 0.110, respectively), HP-$NAP_{58–144}$ after and before treatment (0.201 and 0.210, respectively), and 6× His peptide (0.082). (C) Neutrophil activation ensued after the subtraction of the 6× His value from those of HP-NAPwt (0.152), HP-$NAP_{1–57}$ (0.024) and HP-$NAP_{58–144}$ (0.119).

the protein to bind DNA, whereas Bijlsma *et al.* [27] published positive results, and later Cooksley *et al.* [26], by using immunofluorescence studies, found that an indirect interaction with DNA *in vivo* would be possible. Ceci *et al.* [28] investigated the DNA binding/condensation of HP-NAP at different pH values by using AFM, fluorescence methodologies, and the classic DNA-binding retardation agarose gels. They reported that HP-NAP binds DNA at pH 6.5 and pH 7.0, generating complexes that are too large to migrate into the agarose gel. At pH 7.5 and pH 8.0, the protein is still capable of interacting with DNA, as indicated by the change in mobility of the DNA band in the agarose gels. They postulate that this is in full agreement with the AFM imaging, which shows that, at these pH values, binding of DNA does not entail formation of the large protein–DNA aggregates observed at lower pH values. Additionally, at pH 8.5, HP-NAP does not affect DNA mobility of either linearized or supercoiled plasmids, at least under the buffer conditions studied. Their supporting AFM data at pH 8.0 and pH 8.5 are not quite clear: namely, the protein in both cases seems to be 'in contact' with the DNA, and some molecules (at pH 8.5), as in the case of pH 8.0, are 'free'. If the protein at pH 8.5 did not bind to DNA as shown in the retardation experiments, the AFM imaging would probably be quite different.

The minimal retardation of DNA that is reported for pH 8.0 and shown by agarose gel experiments is not in agreement with that reported by Wang *et al.* [29]. These authors reported a 'strong' interaction of HP-NAP at pH 8.0 that generated complexes too large to migrate into the agarose gel, similar to the complexes generated by the binding of HP-NAP at pH 6.5 and pH 7.0 reported by Ceci *et al.* [28].

The amino acid sequence of HP-NAP exhibits significant similarities with *E. coli* Dps family members, with *Listeria innocua* dodecameric ferritin (Flp), with two Dps-like proteins (Dlp-1 and Dlp-2) from *Bacillus anthracis* [15,31,32], and with *A. tumefaciens* Dps [19]. The absence of the first N-terminal residues of HP-NAP, *B. anthracis* Dlp-1 and Dlp-2, and *Listeria* ferritin, correlates with their inability to form a complex with DNA [7,8,11,19], whereas the short, two-Lys-containing N-terminus of *B. subtilis* MrgA accounts for its binding to DNA [19,30]. In *Streptococcus* mutants, the Dpr (Dps-like peroxidase resistance) protein does not interact with DNA, in accordance with the presence of a long N-terminal tail that does not contain positively charged residues, apart from two Lys residues located near the predicted beginning of the A-helix [21]. Of interest is the formation of a Dps–DNA complex by *Mycobacterium smegmatis* Dps [33]. This protein has a truncated, uncharged N-terminus, but contains an unusually long C-terminus with three Lys and two Arg residues that is thus obviously able to substitute for the N-terminus in the interaction with DNA. The behavior of *Synechococcus* sp. strain PCC 7942 Dps remains unexpected. This heme-binding Dlp is reported to bind DNA [23], despite the absence of Lys or Arg residues in the long N-terminus and the C-terminal extension. In addition, according to Ceci *et al.* [19], Dps from *A. tumefaciens* does not exhibit DNA-binding ability, in spite of the presence of a positively charged N-terminal extension, which is 11 residues shorter than that of the homologous Dps of *E. coli*. From the aforementioned data, the probable interaction between a given Dps and DNA may not be predictable exclusively on the basis of simple sequence analysis of the N-terminus. However, HP-NAP, much like *A. tumefaciens* Dps, protects DNA from oxidative damage due to the ferroxidase activity, despite its inability to bind DNA.

Our data show that HP-NAP does not bind DNA (Fig. 5) but protects it from oxidative damage as a dodecamer (Fig. 6, lanes 2, 4, 6 and 8). In contrast, after destruction of its conformation by replacement of the amino acids that participate in the ferroxidase center, DNA is totally degraded (Fig. 6, lane 11).

The retardation observed by using Hepes at pH 7.5 (Fig. 5A) can probably be attributed to DNA 'unfolding', leading to forms with a lesser degree of supercoiling, and this effect does not seem to be induced by binding of HP-NAP to DNA. The protein protects DNA from destruction by blocking the Fenton reaction, due to iron oxidation, without, however, directly binding to it, at least under the conditions that we used.

All of these above-mentioned controversial observations could be perhaps attributed to different HP-NAP loading techniques or to buffer effects in conjunction with the iron solution.

Therefore, taking into account the above-discussed reports concerning the DNA binding of HP-NAP, we suggest that HP-NAP has a similar function as other Dps family members in protecting cells from oxidative stress damage, and such a role of the protein in the host environment has yet to be investigated.

Another important role of the protein is to activate neutrophils and to stimulate a cellular signal transduction pathway. Its ability to induce these events in the eukaryotic host cells makes it distinct from other members of the Dps family. HP-NAP is chemotactic for neutrophils and monocytes, and it induces ROI production in humans by activating the plasma membrane NADPH oxidase via a signaling pathway involving trimeric G-protein, phosphatidylinositol 3-kinase, Src family tyrosine kinases, and an increase in cytosolic Ca^{2+}. The pattern of events triggered by HP-NAP closely resembles the patterns triggered by heptahelical receptors specific for the chemotactic agonist formyl-Met-Leu-Phe peptide (fMLP), C5a, platelet-activating factor and interleukin-2 [34–36]. Such similarity also strongly suggests that the HP-NAP receptor is a serpentine type of cell surface transmembrane protein, but until now the nature of this receptor has been unknown.

In an attempt to elucidate the region(s) of HP-NAP that interact with cell surface receptor(s), we designed a series of experiments as described under Experimental procedures and presented in Results. After the observation that both HP-NAPwt and HP-NAPmut activate human neutrophils in a similar manner (Fig. 8A, A_{550} 0.234 and 0.214, respectively), we focused on the structure of HP-NAP, and specifically on the structural elements that seem to be exposed and are therefore suitable candidates for the binding of the protein with the receptor. Namely, helices H3 (Leu69–Leu75) or H4 (Lys89–Leu114) or the linking coils (His63–Thr68 and Thr76–Lys83) (Fig. 7), either separately or in conjunction, could be responsible for the activation. After cloning and purification of the N-terminal and C-terminal region, the proteins and their truncated forms were treated with polymixin-coated magnetic beads for LPS removal. Neutrophil activation assays were performed before and after treatment, and the observed absorptions are given in Fig. 8A,B. The quality of the isolated neutrophils was measured before any activation assay under the same conditions, in order to avoid any kind of artefact. Thus, any measured absorption was attributed to neutrophil

activation caused by the added proteins and not to 'preactivated' neutrophils.

Because the receptor is also activated by the fMLP peptide, and the His tags account for additional positive charge on the proteins, a His hexapeptide was synthesized and its neutrophil involvement was investigated.

According to Fig. 8B, the 6× His peptide by itself exhibited an absorption close to that observed for fMLP and HP-NAP$_{1–57}$, but quite different from that observed for HP-NAPwt and HP-NAP$_{58–144}$. According to Tonello *et al.* [12], a large number of basic residues on the HP-NAP dodecamer surface would be responsible for its neutrophil-activating ability. As mentioned above, the positive charge contribution of the His tags would probably account for the higher observed absorption, and therefore a subtraction from all constructs was done. HP-NAP$_{58–144}$ (Fig. 8C) exhibits an absorption that is close enough to that of HP-NAPmut after subtraction of the 6× His value. According to these measurements, the C-terminal region is probably the major receptor activator. Whether the C-terminus alone without the contribution of the N-terminus forms the H3 and H4 helices is not apparent from our studies, and therefore we cannot postulate that the primary sequence itself accounts for the activation. On the other hand, our MD simulation approaches showed that HP-NAPmut forms dimers that are not stable enough to stimulate the formation of a dodecamer.

Our suggestion of the implication of the C-terminal part of HP-NAP in neutrophil activation is also supported by the structural data of Tonello *et al.* [12]. They reported that the α-helices 38–57 and 124–135 form a negatively charged internal surface that is related to the original function of iron storage, and that the presence of a large number of basic residues on the HP-NAP dodecamer surface could be responsible for its neutrophil-activating ability. Indeed, the C-terminal region possesses 13 basic amino acids (Arg and Lys): 11 of them are exposed, and two are within the interior surface. In contrast, the N-terminal region has six basic amino acids (two of them are involved in the formation of the interior surface of the dodecamer).

In conclusion, it seems that neutrophils are mostly activated by the C-terminal region of HP-NAP, and additional studies with site-directed mutagenesis are required in order to identify the amino acids involved, as well as the mechanism of activation. These studies are important for the rational design of new inhibitory molecules against *H. pylori* inflammation, using peptide sequences as scaffolds.

Experimental procedures

Bacterial strains and media

E. coli strain BL21DE3 was grown at 37 °C on LB liquid medium and LB plates containing 50 μg·mL^{-1} ampicillin.

Cloning of the *H. pylori hpnap* gene into the expression vectors pET-11a and pET29c

The *hpnap* gene was amplified by PCR from the *H. pylori* J99 genome using primers HPNAP_up (5′-GCGGAA TTCCATATGAAAACATTTGAAATT-3′) and HPNAP_ low (5′-GCGGGATCCTTAAGCCAAATGGGCTTG-3′), HPNAP_up (5′-GCGGAATTCCATATGAAAACATTTG AAATT-3′) and HPNAP_low (5′-CCGCTCGAGAGCC AAATGGG-3′), for pET11a and pET29c, respectively.

The restriction sites for *Nde*I, *Bam*HI, *Eco*RI and *Xho*I are underlined. The amplified fragments (bp) were digested with the appropriate enzymes (*Nde*I, *Bam*HI, *Eco*RI and *Xho*I), purified with the QIAquick PCR purification kit (Qiagen, Chatsworth, CA), and subsequently cloned into the expression vectors pET11a or pET29c (Novagen) digested with *Nde*I and *Bam*HI. This plasmid was introduced into *E. coli* BL21DE3.

HP-NAP$_{1–57}$ and HP-NAP$_{58–144}$ regions were amplified by PCR for cloning into the pET29c vector, using the primers HPNAP 1–57-up (5′-GCGGAATTCCATATGAAAA CATTTGAAATT-3′) and HPNAP 1–57-low (5′-CCGCTC GAGCCTTTCAGCGA-3′) (XhoI), and HPNAP 58–144-up (5′-GCGGAATTCCATATGATCGTTCAATTAGGA-3′) (*Eco*RI, *Nde*I) and HPNAP 58–144-low (5′-CCGCTC GAGAGCCAAATGGG-3′), respectively. The restriction sites for *Eco*RI, *Nde*I and XhoI are underlined. The amplified fragments were cloned as described above.

In vitro mutagenesis of HP-NAP

One mutant of HP-NAP (HP-NAPmut) was produced using a three-step PCR mutagenesis protocol as described by Picard *et al.* [37]. The codons for His25, His37, Asp52 and Lys134 were substituted by the codon for Ala. The template used was the recombinant plasmid pET11a/hpnap, and the primers used for the PCR were 5′-GGTGCCTTTCACA TTCCACGCGAAGTTATGCACTTTCAT-3′, 5′-AATTTC TTCAGTGGCTTTCGCCACATTGAAAAAATCGGT-3′, 5′-GATCCTTTCAGCGAGATCCGCAAACATGTCCGC AAACTC-3′ and 5′-TTGCAGCATCCAAATGGACGCTT GCAACTTGGCCAATTG-3′ for H25A, H37A, D52A and K134A, respectively. The PCR products were purified, cloned into pET11a, and screened for mutations. The DNA sequences of the resulting mutants were confirmed by nucleotide dideoxy sequencing. HP-NAPmut was cloned in pET11a or pET29c vectors when it was

needed, by using the same primers as for the wild-type protein.

Expression and purification of HP-NAPwt

E. coli BL21DE3 cells harboring the recombinant plasmid were grown in 0.5 L of liquid LB containing ampicillin (50 $\mu g \cdot mL^{-1}$) at 37 °C to an attenuance of 0.7 at 600 nm. After addition of 1 mM isopropyl-β-D-thiogalactopyranoside to induce the transcription of the *hpnap* gene, the culture was incubated for a further 1.5 h.

Cells were harvested (15 000 ***g*** for 20 min), suspended in buffer A (20 mM Tris/HCl, pH 7.5, 500 mM NaCl, 2 M urea), and disrupted by sonication. The lysate was centrifuged at 15 000 ***g*** for 30 min. The supernate was made 98% saturated with respect to ammonium sulfate at room temperature. At this saturation, HP-NAP remains in solution after centrifugation (15 000 ***g*** for 45 min); the supernate was dialyzed overnight against 20 mM Tris/HCl (pH 7.5) and 50 mM NaCl, and then loaded onto a DEAE–sepharose column, equilibrated with the same buffer. HP-NAP was detected in the flow-through fraction, which was subsequently concentrated in an Amicon apparatus (UM10 filter, cut-off 10 000), loaded onto a Sephadex-G200 gel filtration column, and eluted with the same buffer as above. The fractions containing HP-NAP were pooled, concentrated using an Amicon filter (UM10), and stored at −20 °C after controlling the purity of the preparation by 12% SDS/PAGE and staining with silver.

The purification of the recombinant HP-NAP tagged with 6× His was performed by using an Ni–nitrilotriacetic acid affinity column. After cell collection, lysis in buffer A containing 2 M urea, and binding to the column beads, the protein was eluted by using the same buffer made 300 mM with respect to imidazole. The eluate was dialyzed against 20 mM Tris/HCl (pH 7.5) in 50 mM NaCl and stored at −20 °C.

Expression and purification of HP-NAPmut

The procedure followed for growing, harvesting and disrupting cells was the same as for HP-NAPwt, described above. Following centrifugation of the sonicated lysate, the supernate was made 90% saturated with respect to ammonium sulfate at room temperature. At this saturation, HP-NAPmut remained in solution. Following centrifugation (15 000 ***g*** for 45 min), the supernate was dialyzed overnight against 20 mM Tris/HCl (pH 7.5) and 50 mM NaCl, and loaded onto an Ni–nitrilotriacetic acid column (Qiagen) equilibrated with the same buffer. Bound HP-NAPmut was eluted using buffer A containing 300 mM imidazole. The eluate was dialyzed against buffer containing 20 mM Tris/HCl (pH 7.5) and 50 mM NaCl, and its purity was established by subjecting the dialyzed eluate to 12% SDS/PAGE and staining the gel with Coomassie Blue.

Expression and purification of HP-NAP$_{1–57}$ and HP-NAP$_{58–144}$

E. coli BL21DE3 cells harboring the recombinant plasmids pET29c–HP-NAP$_{1–57}$ and pET29c–HP-NAP$_{58–144}$ were grown in 0.5 L of liquid LB containing ampicillin (50 $\mu g \cdot mL^{-1}$) at 37 °C to an attenuance of 0.7 at 600 nm. After addition of 1 mM isopropyl-β-D-thiogalactopyranoside to induce the transcription of the *hpnap* gene, the culture was incubated for a further 1.5 h.

Protein fragment purification of pET29c–HP-NAP$_{1–57}$

Cells were harvested (15 000 ***g*** for 20 min), suspended in a buffer containing 20 mM Tris/HCl (pH 7.5), 500 mM NaCl and 6 M urea, and disrupted by sonication. The lysate was centrifuged at 15 000 ***g*** for 45 min, and the supernate was incubated with Ni–nitrilotriacetic acid beads and eluted by using the incubation buffer including 300 mM imidazole. Owing to the appearance of other contaminating protein bands, the eluate was again dialyzed against 20 mM Tris/HCl (pH 7.5) and 50 mM NaCl, and precipitated by using one ammonium sulfate cut at 40–60% saturation (w/v). The protein fragment remained in solution in a pure form, and was subsequently dialyzed against 20 mM Tris/HCl (pH 7.5), 50 mM NaCl, visualized by 20% w/v SDS/PAGE, and stored at −20 °C in appropriate aliquots.

Protein fragment purification of pET29c–HP-NAP$_{58–144}$

The fragment pET29c–HP-NAP$_{58–144}$ was purified as described above, except that the ammonium sulfate step was eliminated, because after elution from the Ni–nitrilotriacetic acid beads, the preparation was very pure. The eluate was dialyzed against 20 mM Tris/HCl (pH 7.5) and 50 mM NaCl buffer, tested for its purity on 15% SDS/PAGE, separated in aliquots, and stored at −20 °C.

LPS removal with polymixin B-coated magnetic beads

The purified His-tagged entire proteins HP-NAPwt and HP-NAPmut, and the fragments HP-NAP$_{1–57}$ and HP-NAP$_{58–144}$, were dialyzed against NaCl/P_i (pH 7.5) and treated with polymixin B-coated magnetic beads (25 $mg \cdot mL^{-1}$; Chemicel, Berlin, Germany) in order to remove LPS. Ten milligrams of LPS removal beads per protein were transferred into 1.5 mL reaction tubes (Eppendorf) and washed three times with 1 mL of NaCl/P_i. To bind LPS, protein solutions were added to the beads and mixed constantly for 30 min at 4 °C. Afterwards, the tubes were placed in a magnet, and the clear protein solutions

were transferred into fresh reaction tubes. The protein solutions were filtered with 0.2 μm syringe filters, and their concentration was determined by measuring the absorbance at 280 nm.

Dodecamer formation

The dodecamer formation of HP-NAP was first demonstrated by Sephacryl-200 gel chromatography. The protein, after its purification, as described above, was applied to a Sephacryl-200 column pre-equilibrated with 20 mM phosphate buffer (pH 7.5) and 150 mM NaCl. The flow rate was 0.125 mL·min^{-1}, and the absorbance was measured at 280 nm. The fractions – 4 mL each – were analyzed on 12% SDS/PAGE. The elution profile of the protein was correlated with markers with known molecular masses, such as aldolase (160 kDa), albumin (68 kDa), and cytochrome *c* (14 kDa), that were separated under the conditions described above.

In addition, the formation of dodecamers was investigated by a simple method as described by Stern *et al.* [38], although it has been developed for proteins containing SH groups (HP-NAP has no SH groups). The SDS concentration was 0.5% w/v in the gels used for separating and stacking gels, and 2% in the sample buffer.

The protein samples of HP-NAPwt and HP-NAPmut were subjected to 12% SDS/PAGE in the absence of sulfhydryl reagents in the sample buffer and without being heated prior to electrophoresis.

Iron uptake and ferroxidase activity

Recombinant HP-NAP was incubated for 18 h in a solution containing 1% thioglycolic acid and 0.1 M sodium acetate (pH 5.5) [39]. The Fe^{2+} was chelated by the addition of 2,2-bipyridine in excess to the reaction, and the solution was dialyzed exhaustively against 0.1 M Hepes (pH 7.0).

The kinetics of iron uptake were recorded as described previously [40], with partial modification. Recombinant HP-NAP and BSA (control protein), each 20 μg·mL^{-1}, were incubated for 10 min at room temperature in 0.1 M Hepes (pH 7.0) containing 1 mM ferrous ammonium sulfate. The amber color change was measured at 310 nm.

Ferroxidase activity assays were routinely performed as previously described [41], at room temperature, by using ferrous ammonium sulfate as the electron donor and 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) as a chelator that specifically detects the Fe^{2+} remaining at the end of the reaction. Each assay mixture (0.2 mL) contained 50 mM sodium acetate buffer (pH 5), and 0.35–1.05 mg·mL^{-1} HP-NAP. The reaction was started by adding $Fe(NH_4)_2SO_4$ to a final concentration of 50 μM. Reactions were quenched after 1 h by adding 3.25 mM ferrozine, and Fe^{2+} oxidation was determined by measuring the absorbance of residual Fe^{2+}–ferrozine at 570 nm.

Gel retardation assays

The DNA-binding activity of HP-NAP in the iron-loaded form was assessed by gel shift assays using a supercoiled recombined plasmid with the ribosomal protein S14 from *Thermus thermophilus* pTZ-S14 DNA (20 nM). The buffers were 20 mM phosphate and 50 mM NaCl (pH 6.5), and 20 mM Hepes and 50 mM NaCl (pH 7.5). The HP-NAP was loaded with 0.5 mM $Fe(NH_4)_2SO_4$. The plasmid DNA was incubated with HP-NAP (60–200 nM) at 37 °C or at 4 °C for different incubation times (from 60 to 120 min). The incubation mixtures were loaded onto 1% agarose gels, and subjected to electrophoresis for 30 min, in TBE buffer (89 mM Tris, 0.45 M H_3BO_3, 2 mM EDTA, pH 8.0), and the gels were stained with ethidium bromide.

DNA protection assay

DNA protection from oxidative damage was assessed by *in vitro* using the *TthS14* gene purified by a Qiagen kit. The reaction mixture contained the following reagents in a total volume of 40 μL at the final concentration stated: 20 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.5 mM $Fe(NH_4)_2SO_4$, 1.25 μg·mL^{-1} DNA (*TthS14* gene), and 19 nM HP-NAP. The samples were incubated for 15, 30, 45 and 60 min at room temperature, and the products were visualized on agarose gel 1% w/v. In one sample (control), 12.5 mM EDTA was added to chelate Fe^{2+}, preventing the degradation of DNA.

Neutrophil isolation and activation

Human neutrophils were prepared from buffy coats of venous blood of healthy donors as previously described [42]. The procedure is a modification of the method of Boyum [43], and includes centrifugation of cells in Ficoll medium and sedimentation of the mixture in T-500 dextran solution 6% w/v. Erythrocytes remaining in the granulocyte fraction were removed by lysis in a 0.8% w/v solution of NH_4Cl in H_2O. After incubation in NH_4Cl for at least 10 min, the cells were centrifuged at 400 ***g***, and the supernate was discarded. The lysis and centrifugation were repeated until the preparation was free of erythrocytes. This procedure usually results in granulocyte fractions with neutrophil contents of more than 95%.

The amount of superoxide anions produced by neutrophils was measured via the SOD-inhibitable reduction of cytochrome *c*. Briefly, neutrophils (10^6) were incubated with 1 mg·mL^{-1} cytochrome *c* in the presence of 1 μM HP-NAP with or without 20 μg·mL^{-1} SOD at 37 °C for 30 min, and then subjected to rapid centrifugation. The

absorbance of the supernatant was determined spectrophotometrically at 550 nm. The amount of superoxide anions was measured as the difference in absorbance of those incubated with or without SOD. Each sample was assayed in triplicate [10].

Synthesis of H_2N-His-His-His-His-His-His-OH

Synthesis was performed by solid-phase methodology on a 2-clorotrityl chloride resin [44], using the Fmoc/tert-Butyl chemistry [45]. Fmoc-protected His was used, with the trityl group as side-chain-protecting group. In summary, the Fmoc group was removed with 25% piperidine in *N,N*-dimethylformamide. Activation of each amino acid was performed *in situ*, using diisopropylcarbodiimide/1-hydroxybenzotriazol in *N,N*-dimethylformamide. Couplings were performed with 3 : 3.3 : 4.5 molar excess of Fmoc–amino acid/diisopropylcarbodiimide/1-hydroxybenzotriazol respectively. The completeness of the reaction was monitored by the Kaiser test [46]. Treatment of the peptidyl resin with trifluoroacetic acid/water/triethylsilane (95 : 2.5 : 2.5, v/v/v) (15 $mL \cdot g^{-1}$ peptide resin) for 3.5 h afforded the desired product. The solvent was removed on a rotary evaporator, and the product was precipitated as a white solid by addition of cold and dry diethyl ether. The crude peptide was purified by gel filtration chromatography on Sephadex G-10 using 15% acetic acid as the eluent. Final purification was achieved by semipreparative HPLC (Mod.10 ÄKTA; Amersham Biosciences, Piscataway, NJ, USA) on Supelcosil C18 (5 μm particle size, 25 cm × 8 mm; Sigma-Aldrich, St Louis, MO, USA), with a linear gradient from 0% to 40% acetonitrile containing 0.1% trifluoroacetic acid for 30 min at a flow rate of 1.5 $mL \cdot min^{-1}$, and UV detection at 214 and 230 nm. The appropriate fractions were pooled and lyophilized. An analytical HPLC column equipped with a Nucleosil 100 C18 column (5 μm; 25 cm × 4.6 mm; Agilent Technologies, Waldbronn, Germany) produced a single peak with at least 98% of the total peak integrals. ESI MS (Micromass-Platform LC instrument; Waters-Micromass Technologies, Milford, MA, USA) gave a mass that was in agreement with the expected mass.

MD simulations

In order to study and illustrate the structural properties of the mutated HP-NAP and to test our hypothesis that the mutations lead to a reduced ability to associate and form dodecamers, MD simulations were performed on dimers of the HP-NAP monomers of the wild-type as well as the mutated type immersed in a water (TIP3) sphere of 40 Å radius. The dimer AD-wt was formed from chains A and D in an antiparallel configuration as they are found in the resolved dodecameric crystal structure 1JI4.pdb, whereas the dimer AD-4mut was formed by mutating His25 → Ala, His37 → Ala, Asp52 → Ala and Lys134 → Ala, using VMD software [47]. In addition to the above, Glu56 was also mutated to Ala (not included in the experiments), giving the model AD-5mut. The crystallographic dimer AD holds two Fe^{2+} very closely, one between A-Glu56, A-Asp52, and D-His25, and its symmetry equivalent between D-Glu56, D-Asp52, and A-His25. For His25 and His37, the neutral form was used with the proton at Nδ1, whereas for all other His residues, the proton is at Nε1. This is reasonable because of the presence of the ferrous ions in the vicinity of His25 and His37. The systems AD-wt, AD-4mut and AD-5mut were then neutralized by adding 10 Na^+ (eight in AD_5mut) in the water environment, and this was followed by energy minimization and a 100 ps equilibration at 300 K for only the water part of the systems. Finally, the dimers were equilibrated for 1.5 ns at 300 K (with a Langevin Thermostat). The Fe^{2+} was treated as a simple divalent ion, without considering its coordination sites. The crystal structure of the 'ferroxidase site' (Protein Data Bank: 1JI4) (His25:NE2, Glu56:OE2, Asp52:OD2, UnX) does not show the typical tetrahedral or hexahedral iron coordination geometry, as this is known from the transition metal complexes, and no water molecules bind to the Fe^{2+}. Hence, we assume a certain amount of Fe^{2+} flexibility, which is more consistent with trivial ionic interactions than with a typical coordination. At the end of each equilibration, we averaged the final 50 ps to form a more representative structure, which we used for further analysis. Energy minimization and MD simulations were performed using the software NAMD [48] with the CHARMM27 force field for proteins and nucleic acids. The thermodynamic analysis was performed using the computer program STC [49].

Acknowledgements

This work was supported by a grant from the General Secretariat of Research and Technology, Ministry of Development of Greece, by the Program HERAKLITOS. The authors gratefully acknowledge Emeritus Professor I. Georgatsos for critical reading of the manuscript, and Dr D. Triantafillidou and Dr K. Anagnostopoulos for editing assistance.

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Supplementary material

The following supplementary material is available online:

Doc. S1. Calculation of the ratio of the dimerization equilibrium constants of mutant and wild-type *Helicobacter pylori* neutrophil-activating protein.

This material is available as part of the online article from http://www.blackwell-synergy.com

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Journal of Surgical Research **146,** 149–158 (2008)
doi:10.1016/j.jss.2007.06.011

RESEARCH REVIEW

New Aspects of *Helicobacter pylori* Infection Involvement in Gastric Oncogenesis

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Submitted for publication January 14, 2007

Gastric adenocarcinoma not located in the cardia still remains second only to lung cancer as the leading cause of cancer-related mortality worldwide, whereas adenocarcinoma of the cardia and gastroesophageal junction has been rapidly rising over the past two decades. Gastric malignancy can be subdivided into diffuse and intestinal pathologic entities that have different epidemiological and prognostic features. Various genetic and environmental factors lead to either abnormal gene overexpression or inappropriate expression of normal genes, whose products confer the malignant phenotype. Advances have been made in genetic changes mostly of the intestinal type; its development is probably a multistep process, as has been well described in colon carcinogenesis. Oncogene overexpression, tumor suppressor loss, and defective DNA mismatch repair is associated with gastric cancer. The most common genetic abnormalities tend to be loss of heterozygosity of particularly tumor suppressor p53 gene or "*adenomatous polyposis coli*" gene. The latter leads to gastric carcinogenesis through changes related to E-cadherin-catenin complex, which plays a critical role in normal tissue architecture maintenance. Mutation of any of its components results in loss of cell-cell adhesion, thereby contributing to malignancy. Putative trophic factors have also been involved in gastric oncogenesis. E-cadherin/CDH1 gene germline mutations have been recognized in families with an inherited predisposition to diffuse-type malignancy. This review focuses mainly on *Helicobacter pylori* infection involved in gastric carcinogenesis through various mechanisms, including repopulation of the stomach with bone marrow-derived stem cells that may facilitate gastric cancer progression, thereby necessitating eradication of this bacterium.

Key Words: **gastric cancer; epidemiology; *H. pylori*; oncogenes; tumor suppressor genes; microsatellite instability; apoptosis.**

INTRODUCTION

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

Importantly, the remarkable decrease in the adenocarcinoma of the stomach in the United States during the last 70 years has occurred primarily for the intestinal type associated with *Helicobacter pylori* (*H. pylori*) infection (class I carcinogen, WHO), achlorhydria, and intestinal metaplasia. The incidence of the diffuse-

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0022-4804/08 $34.00

type gastric cancer has remained constant over time. In contrast, apart from the proximal gastric cancers, there has been a relative increase in distal esophageal adenocarcinomas, particularly those associated with Barrett's esophagus [3]. A small but significant occurrence of cancer in gastric remnants has been reported in patients who have undergone gastrectomy for benign disease. A latency period of approximately 15 to 20 years has been noted before significantly increased risk [4]. Data collected worldwide demonstrate that a consistent predominance of gastric cancer in males (black and white) is seen worldwide, with about a 2:1 male:female ratio. In countries with a high incidence of gastric malignancy, the age at diagnosis tends to be a decade earlier. When gastric cancer affects younger patients, the male:female ratio is close to 1, there is a high preponderance of blood group A, as well as a family history of cancer and a higher incidence of the diffuse-type gastric cancer than the intestinal type [2]. Mass screening programs in high-risk regions such as Japan have helped identifying and diagnosing gastric malignancies earlier, with some decrease in mortality rates, although significant improvements in tests for earlier diagnosis are needed [4].

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

CLASSIFICATION—ETIOLOGY

Based on Lauren classification, gastric cancer can be subdivided into two distinct pathologic entities, diffuse (infiltrating or scattered malignant cells or islands of cells) and intestinal (gland-forming or expansive) that have different epidemiological and prognostic features (Table 1) [5]. The diffuse type involves two different subtypes: "pure" (poorly differentiated carcinoma lacking any glandular structure) and "mixed" type (coexistence of poorly differentiated carcinoma and intramucosal glandular structure). Diffuse gastric cancers exhibit signet-ring cells that aggressively infiltrate tissues, with nuclei that are eccentrically displayed by unsecreted mucus, and often presenting as linitis plastica. Apart from the same frequency throughout the world, diffuse-type gastric cancers present more diffusely in the stomach and earlier in life, arise without identifiable precursor lesions, and tend to spread contiguously into the peritoneum, whereas intestinal-type gastric malignancies tend to spread hematogenously and are accompanied by a worse prognosis than the intestinal type. Intestinal-type malignancy is more closely linked to environmental and dietary risk factors and is the type of cancer that is now declining worldwide. The importance of distinguishing these two main histopathologic types of gastric cancer is highlighted by the finding of specific genetic changes associated with the different types [4].

It is now thought that the development of the intestinal-type malignancy is likely a multistep process, as has been well described in the pathogenesis of colon cancer. The progressive accumulation of genetic changes in both oncogenes and tumor-suppressor genes parallels the clinical and histopathologic progression from normal colonic epithelium through benign adenomas to frank colon cancer. In particular, the progression from adenoma to colon carcinoma results from the accumulation of molecular genetic alterations involving mainly three factors: activation of oncogenes, inactivation of tumor suppressor genes, and abnormalities in genes involved in DNA mismatch repair [6]. The contention that the pathogenesis of the intestinal-type gastric cancer is also a multistep process, comprising gastric mucosal metaplasia-dysplasia-carcinoma sequence, is supported by the evidence that both atrophic gastritis and intestinal metaplasia are found in higher incidence in patients with intestinal-type cancer and in areas with high incidence of gastric cancer

TABLE 1

Epidemiologic and Prognostic Features of the Two Distinct Types of Gastric Cancer

	Diffuse-type	Intestinal-type
Age	Younger ages	Older ages
Environmental and dietary risk factors	Not associated?	Associated
Incidence	Constant over time	Declining
Appearance	Infiltrating or scattered malignant cells or islands of cells (signet-ring cells)	Gland-forming or expansive
Sybtypes	Pure: Absence of any glandular structure Mixed: Coexistence of glandular structure	-
Precursor lesions	Not identifiable	Multistep metaplasia-dysplasia-carcinoma sequence
Spreading	Contiguously into the peritoneum and via the lymphogenous route	Hematogenously
Prognosis	Worse	Less unfavorable

TABLE 2

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

	Colon	Stomach	Esophagus
Oncogenes			
Ras	+	+	
c-myc	+	+	+
c-erb B1		+	
c-erb B2	+	+	+
hst-1	+	+	
trk	+		
c-raf	+		
c-scr	+		
c-myb	+	+	
c-yes			
c-mos			
c-fos			
Tumor suppressor genes			
p53	+	+	+
APC	+	+	+
DCC	+	+	+
MCC	+	+	+
DPC4	+		
DNA mismatch repair genes			
hMSH2, hMLH1	+	+	+

APC = adenomatous *polyposis coli*; MCC = mutated in colon cancer.

[4, 5]. This multistep model of gastric cancer postulates that there is initially an inflammation, caused by *H. pylori* infection, as well as by exposure of toxins (preserved foods, high-salt diet, bile salts), which can lead to the development of chronic active gastritis. In a subset of these patients, this inflammatory process leads to the development of atrophic gastritis, followed by intestinal metaplasia, dysplasia, and ultimately early and advanced gastric cancer. It is considered that all stages prior to the development of high-grade dysplasia are potentially reversible, although this is still controversial. Unlike the case of colon cancer, the precise genes involved in each step of this progression are still not accurately defined. This is due to the fact that the premalignant stages of gastric cancer are not as readily identifiable endoscopically for prospective study compared with colon malignancy. In addition, several gastric tumors are very heterogeneous, containing a large proportion of normal stromal cells that may confound genetic analysis. Moreover, while there is increasing evidence that a genetic predisposition, in at least a subset of patients, plays an important role in gastric cancer, characterization of the timing of specific gene mutations in gastric cancer is made difficult at best. At present time, it still remains tentative whether the diffuse-type gastric cancer follows an analogous histopathologic progression [2].

GENETIC FACTORS AND *H. PYLORI* INFECTION

The accumulation of multiple genetic alterations, leading to tumor suppressor loss, defective DNA mismatch repair, and oncogene overexpression, is associated with tumors of the gastrointestinal tract, including gastric cancer (Table 2, Table 3), [6, 7].

Genetic Alterations Leading to Tumor Suppressor Loss and Defective DNA Mismatch Repair

Gastric carcinomas are believed to evolve from native gastric mucosa or intestinal metaplastic mucosa that undergo genetic and epigenetic alterations involving either the suppressor pathway (defects in tumor suppressor genes) or the mutator pathway (defects in DNA mismatch repair genes) [8]. A progress has been made in our understanding of the genetic changes that occur mostly in the intestinal-type gastric cancer.

The most frequent genetic abnormalities found tend to be loss of heterozygosity (LOH) of previously described tumor suppressor genes. Of note, mutations that disrupt the biological function of these genes have been found in association with cancers of the stomach, as well as esophagus and colon. The gene that has garnered the most attention is the tumor suppressor p53. This is a nuclear oncosuppressor protein involved in the maintenance of genomic integrity. DNA damage results in the increased expression of p53, which then causes G1 arrest in the actively cycling cells. It can then induce factors that allow DNA repair to occur or, if the damage is too great, factors which cause apoptosis [6]. Early studies reported that LOH (60% to 70%) and mutations (38% to71%) of the p53 gene are quite

TABLE 3

Genetic Changes in Gastric Adenocarcinoma

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

APC = adenomatous *polyposis coli*; COX-2 = cyclooxygenase-2; HGF/SF = hepatocyte growth factor/scatter factor; VEGF = vascular endothelial growth factor; MSI = microsatellite instability.

frequent in gastric cancer. In addition, p53 mutations are also observed in intestinal metaplasia (38%) and gastric dysplasia (58%), suggesting that mutations of the p53 gene may be an early event and perhaps work together with RAS oncogene in the pathogenesis of gastric cancer [9]. Further evidence for a role of p53 in the early stages of gastric cancer development comes from studies in mice that are hemizygous for p53, which display an increased proliferative response to *H. pylori* infection compared with wild-type mice. Increased proliferation is correlated with an increased risk in developing gastric malignancy. From another viewpoint, recent studies indicate that the inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), and the tumor suppressor p53 are fundamental play-markers of the angiogenic process. Overexpression of iNOS and VEGF has been shown to induce angiogenesis in tumors, whereas p53 suppresses angiogenesis by down-regulating VEGF and iNOS. On the other hand, mutations of the p53 gene have been thought to up-regulate VEGF and possibly iNOS [10]. In this regard, *H. pylori* infection induces p53 mutations [11], up-regulation of VEGF [12, 13], and iNOS expression and subsequent DNA damage as well as enhanced anti-apoptosis signal transduction [14], thereby contributing to gastric carcinogenesis (Table 4).

LOH at the 5q allelic locus, the site of the "*adenomatous polyposis coli*" (APC) and "mutated in colon cancer" genes, occurs in over a third of gastric tumors but not in gastric dysplasia, with LOH being more common in the intestinal type regardless of stage [2]. Focusing on APC, this gene resides on the long arm of chromosome 5. Inactivation of both copies of the APC gene has been found to be the "gate-keeping" event for the initiation of colorectal neoplasia. APC gene abnormalities may lead to disruption of normal cell-cell adhesion through altered association with molecules called catenins and cell adhesion molecule E-cadherin, a transmembrane glucoprotein that binds catenins. In particular, E-cadherin connects to the actin cytoskeleton through α- and β-catenin to establish cell polarity and mediates homophilic cellular interactions, indicating the involvement of E-cadherin in the formation of cell junctions and the maintenance of epithelial integrity [15]. Therefore, the E-cadherin-catenin complex is an important element for maintaining intercellular adhesion and plays a critical role in the maintenance of normal tissue architecture. Mutation of any of its components is believed to result in loss of cell-cell adhesion, thereby contributing to neoplasia, and is associated with poor differentiation and increased invasiveness of carcinomas [6]. Mutations or losses of the APC gene lead to susceptibility in colonic neoplasms in patients with familial adenomatous polyposis, and somatic mutations of the APC gene occur in more than two thirds in sporadic colorectal carcinomas and adenomas including the smallest dysplastic lesions [6]. Additional evidence supporting a role for APC in the pathogenesis of some forms of gastric cancer comes from the fact that familial adenomatous polyposis patients have a 10-fold higher risk of developing gastric cancer compared with the general population. Mutations of APC gene occur in up to 20% of sporadic gastric cancers and gastric adenomas, mainly in well-differentiated intestinal gastric cancers in which up to 60% may have APC mutations [2]. The mechanism of action of the APC gene is to sequester and inactivate cytoplasmic β-catenin preventing the formation of β-catenin/lymphoid enhancer factor, which acts as a growth-promoting transcription factor. It is known that β-catenin plays two distinct roles, in intercellular adhesion by E-cadherin already mentioned, and in transcriptional activation via TCF/lymphoid enhancer factor. Theoretically, the former role is tumor-suppressive, while the latter is oncogenic. β-Catenin mutations, preventing its inactivation by APC, are also found in an additional 16% to 27% of sporadic intestinal-type cancers. It is important to note that in intestinal-type gastric cancers β-catenin mRNA levels are greatly enhanced [16]. High intranuclear levels of β-catenin protein play an important role in early tumor growth and may initiate the invasive processes in intestinal-type gastric carcinoma. In addition, β-catenin expression is lost in a subgroup of primary gastric cancers, is frequently absent in metastases and exhibits

TABLE 4

Summary of the Mechanisms by Which *Helicobacter* pylori May Promote Gastric Oncogenesis

H. pylori:
- i) Induces p53 mutations
- ii) Up-regulates VEGF expression
- iii) Up-regulates iNOS expression
- iv) Disrupts E-cadherin/catenin-containing adherens junctions
- v) Synergizes with chronic hypergastrinemia contributing to parietal cell loss
- vi) Induces gastrin
- vii) Up-regulates COX-2
- viii) Activates NF-κB
- ix) Increases PGE_2 synthesis
- x) Activates c-Met
- xi) Induces IL-8, acting as angiogenic factor
- xii) Up-regulates matrix metalloproteinase-9 expression
- xiii) Causes migration of bone marrow-derived stem cells to stomach
- xiv) Is associated with development of atrophic gastritis
- xv) Up-regulates mainly Ki-67, secondarily Bcl-2, and Bax expression, leading to increased proliferation rate
- xvi) Induces apoptosis of T lymphocytes with subsequent immune evasion
- xvii) Up-regulates anti-apoptotic Bcl-x_L in BLs, associated with prolonged BLs survival

VEGF = vascular endothelial growth factor; iNOS = inducible nitric oxide synthase; COX-2 = cyclooxygenase-2; NF-κB = nuclear factor-κB; PGE_2 = prostaglandin E_2; IL-8 = interleukin-8; BLs = B lymphocytes.

nuclear localization in cancers with either β-catenin or APC gene mutations. The loss of β-catenin expression in metastatic gastric cancers may result from hypermethylation of the β-catenin promoter. Therefore, the high frequency of either APC or β-catenin mutations and/or LOH of the APC locus reported suggest an important role of APC in the pathogenesis of gastric cancer [2, 16]. In this regard, *H. pylori* infection induces disruption of E-cadherin/catenin-containing adherens junctions on gastric epithelium, leading to gastric oncogenesis (Table 4) [17].

A number of other genes have been reported to be either mutated or suppressed in gastric cancer, although their relative significance in the pathogenesis of gastric cancer remains to be determined (Table 3) [2, 18].

Genetic Alterations Leading to Oncogene Overexpression

A variety of oncogenes and the proteins encoded by them also appear to play a significant role in the pathogenesis of gastric cancer. These proteins constitute at least four distinct groups: (1) peptide growth factors that may be secreted into the extracellular milieu; (2) protein kinases, including receptor and nonreceptor tyrosine kinases and cytoplasmic serine/threonine kinases; (3) signal transducing proteins associated with the inner cell membrane surface (membrane-associated G proteins that regulate generation of cyclic nucleotides); and (4) nuclear transcriptional regulatory proteins [nuclear factor-κB (NF-κB)] [6].

HYPERGASTRINEMIA AND *H. PYLORI* INFECTION

The best studied and most common oncogene alteration in colonic neoplasm involves the RAS oncogene, which also works together with p53 gene mutation in gastric carcinogenesis [9] and up-regulates the gene expression of gastrin. The latter is an oncogenic growth factor contributing to gastric and colon carcinogenesis [6, 19]. Noteworthy, both low acid secretion and endogenous hypergastrinemia, especially in the elderly, may play an important role in differentiated and undifferentiated gastric carcinomas. Chronic hypergastrinemia in mice can synergize with *H. pylori* infection and contribute to eventual parietal cell loss and progression to gastric cancer [20]. The gastric cultured epithelial cells exhibit the expression of gastrin receptors, and gastrin shows antiapoptotic activity through the up-regulation of Bcl-2 and survivin. Moreover, gastrin stimulates the gene and protein expression of cyclooxygenase (COX)-2 and hepatocyte growth factor (HGF) in human cultured gastric cancer cells, thereby contributing to tumorigenesis [19]. In this regard, *H. pylori* infection may contribute to gastric carcinogenesis via induction of gastrin and COX-2 that may account for the stimulation of tumor growth, angiogenesis, and reduction in apoptosis (Table 4) [19]. Therefore, *H. pylori* positive patients developing gastric or colon cancer should be considered for *H. pylori* eradication to reduce the *H. pylori* provoked hypergastrinemia and COX-2 overexpression in the tumor tissue.

COX-2 AND *H. PYLORI* INFECTION

COX is the key enzyme in conversion of arachidonic acid to prostanoids. Two COX genes have been cloned, and expression of COX-2 mRNA and protein has been shown to be elevated in several human malignancies and in animal models of carcinogenesis. Moreover, recent evidence has implicated COX-2 in gastric, esophageal, and colorectal carcinogenesis. Indeed, increased COX-2 expression was noticed in gastric carcinomas, Barrett's esophagus and esophageal adenocarcinomas, and colorectal adenomas and carcinomas [21]. COX-2 appears to be mutagenic and tumorigenic in vitro. Moreover, COX-2 overexpression may inhibit apoptosis and increases invasiveness of malignant cells [21]. In particular, COX-2 is expressed by the neoplastic cells in the intestinal-type gastric adenocarcinoma and by precarcinogenic (dysplastic) lesions leading to the development of gastric cancer, and its overexpression is associated with lymphatic metastasis, tumor invasion, and differentiation of gastric carcinoma [22]. CagA(+) *H. pylori* infection could up-regulate the expression of COX-2 in gastric cancer in humans [22]. Furthermore, *H. pylori* infection might activate NF-κB, an oxidant-sensitive transcription regulator of inducible expression of inflammatory genes such as COX-2, which regulates human gastric cancer cell growth and proliferation. Thus, oxidant-sensitive transcription factor NF-κB may play a novel role in the expression of COX-2 by *H. pylori* stimulation in gastric cancer cells [23].

Besides, COX-2 overexpression enhances prostaglandin (PG) synthesis and the importance of prostaglandins (PGE_2) in the progression of a chronic inflammation or neoplasia has long been recognized. Although the release of these compounds in response to tissue injury seems to be a key event in the reparative process and inflammatory response, it is becoming clear that they are implicated in cell proliferation and inhibition of immune surveillance; therefore, overproduction of PGs could favor malignant growth [21]. Specifically, synthetic machinery and receptors for PGE_2, prominently expressed by T lymphocytes in gastric mucosa at the boundary of normal mucosa with tumor cells may play a central role in prostanoid-driven tumorigenesis of this tissue [24]. In addition, binding of HGF to its receptor (c-Met) regulates gastric cancer progression and metastasis, up-regulates the expression of COX-2 gene, and increases PG synthesis in gastric mucosa cells [25]. Importantly, *H. pylori*, apart from inducing COX-2 expression, also increases PGE_2 synthesis [26], and the increased levels of PGE_2 in the presence of

cagA+ infection could be an important factor by which cagA+ strains enhance the gastric mucus layer protective functions leading to established colonization, gastritis and increased risk of gastric cancer [27]. On the other hand, inhibition of COX-2 prevents growth of gastric cancer xenografts in nude mice, and aspirin use (which inhibits both COX-1 and COX-2) decreases the risk of developing gastric cancer [2]. Therefore, applying COX-2 selective (or nonselective) inhibitors reduces inflammation, suppresses carcinogenesis in the gastrointestinal tract, and might be an effective and promising way to prevent *H. pylori*-related gastric cancer [22, 28].

However, in the setting of prophylaxis, clinical use of COX-2-selective compounds has ignited strong debates regarding potential side effects, most notably those within the cardiovascular system such as myocardial infarctions, strokes, and elevation in blood pressure [29].

GROWTH FACTORS AND *H. PYLORI* INFECTION

The c-Met gene, a proto-oncogene member of the tyrosine kinase growth factor receptors, is amplified in 10.2% and overexpressed in 46.1% of gastric cancers. Its ligand, HGF/scatter factor (HGF/SF), is also overexpressed in 67% of gastric cancers [2]. Amplification of the c-Met gene is associated with increased depth of tumor invasion, lymph node and liver metastases, and decreased survival [2]. *H. pylori* activates the c-Met, promoting gastric cancer (Table 4) [30]. Moreover, a higher frequency of c-Met expression is observed in α-fetoprotein-producing gastric cancer and is associated with decreased apoptosis, high incidence of liver metastasis, and poor prognosis. A higher expression of c-Met might be one explanation for the poorer prognosis of α-fetoprotein-producing gastric cancers because HGF and its receptor, c-Met, are known to induce mitosis and cell movement, and to promote tumor progression [31, 32].

ANGIOGENESIS AND *H. PYLORI* INFECTION

Amplification and/or overexpression of putative trophic factors have also been observed in gastric cancer. VEGF is a known angiogenic factor that promotes neovascularization of tumors, generally increasing the risk of invasion and metastases. Noteworthy, VEGF is overexpressed in up to 54% of gastric cancers and correlates with the depth of invasion, the staging of gastric carcinoma, an increased risk of lymph node and liver metastases, and with disease recurrence [2, 33]. In general, the activation of coagulation, angiogenesis, and inflammatory cytokines are considered to be related to tumor growth and metastasis. More specifically, in gastric cancer the plasma levels of VEGF and interleukin (IL)-6 are markedly increased in patients with stage IV disease and, thus, they might be useful for identifying metastatic gastric patients [33]; IL-6 may play a role in the angiogenesis of gastric carcinoma via modulation of VEGF. Furthermore, IL-8 (induced by *H. pylori* infection) acts as an angiogenic factor for human gastric carcinomas, up-regulates matrix metalloproteinase-9 expression, and increases invasive activity of gastric carcinoma cells (Table 4). Indeed, the IL-8 level in the neoplasms correlates significantly with the depth of invasion, venous and lymphatic invasion. The chemokine IL-8 (CXCL8) appears to exert potent angiogenic properties on endothelial cells through interaction with its cognate receptors CXCR1 and CXCR2 [34]. Relevant studies have shown than IL-8 directly enhances endothelial cell proliferation, survival, and metalloproteinase expression in CXCR1- and CXCR2-expressing endothelial cells, and regulates angiogenesis [20]. IL-8 and VEGF (contributing to *H. pylori*-related gastric carcinogenesis) [12] may be independent and important prognostic factors in human gastric carcinomas [35]. Moreover, the expression of iNOS and VEGF is closely related to tumor angiogenesis and is involved in the advancement and the lymph node metastases. Important to note is that the association between high blood levels of VEGF and poor prognosis in cancer does not depend only on VEGF-induced stimulation of the neovascularization, but also on VEGF-related immunosuppression (VEGF inhibits dendritic cell maturation and lowers the antitumor cytokine IL-12). Thus, it would appear that VEGF, with or without the combination of IL-6, IL-12, and iNOS levels, may play a role in the development of advanced gastric cancer, and therapy with VEGF antibodies may be a probable therapeutic strategy against human *H. pylori*-related gastric cancer [36].

STEM CELLS AND *H. PYLORI* INFECTION

Recently, Houghton et al. [37, 38] showed that *H. pylori*-induced inflammation in mice caused migration of stem cells originating from bone marrow to the stomach, where they subsequently developed gastric tumors (Table 4). These stem cells progress through metaplasia and dysplasia to intraepithelial cancer, suggesting that epithelial cancers can originate from bone marrow-derived stem cells (BMDCs). In this regard, we recently conducted a pilot study (Kountouras et al., unpublished data) using tissue sections of biopsies of human gastric cancer in which *H. pylori* bacteria were detected by Cresyl violet staining (Fig. 1). Moreover, stem cells and neovessels were detected by immunohistochemical method using a monoclonal antibody anti-CD34; CD34 is a surface glycoprotein expressed on hematopoietic stem cells and is used as an important marker of these cells and neovessels (Fig. 2). In addition, cyclin D1 involved in the regulation of cell proliferation was also detected by immunohistochemical method (Fig. 3). Therefore, it would be reasonable

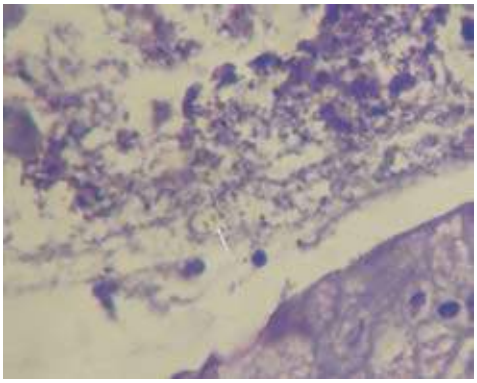

FIG 1. Positive cresyl violet stain for *Helicobacter pylori* bacteria (arrow) at gastric cancer tissue of a patient with gastric carcinoma (×400). (Color version of figure is available online.)

to speculate that chronic infection of C57BL/6 mice and humans with *H. pylori* induces repopulation of the stomach with BMDCs that may facilitate gastric cancer progression as well as colon cancer progression [39]. These findings present a new way of thinking about the pathogenesis of upper gastrointestinal malignancy. The observation that BMDCs are the origin of *H. pylori*-induced gastric cancer can be also combined with supporting observations of BMDCs in other tumors such as Barrett's esophageal adenocarcinoma, Kaposi sarcoma, or benign and malignant tumors of skin [38]. However, more work is needed to show that the BMDCs move into areas of *H. pylori* chronic injury or inflammation with long-term malignant consequences.

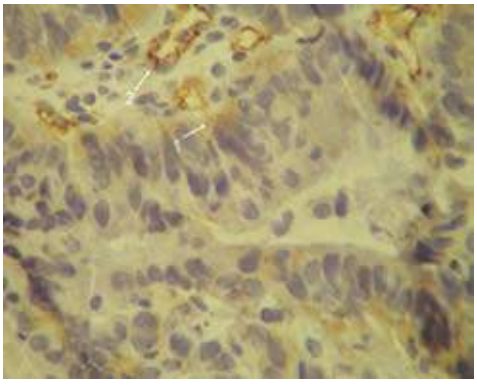

FIG 2. Cytoplasmic positivity in gastric cancer cells in another representative tissue region of the same *Helicobacter pylori* positive patient, for CD34 staining (1). Note positivity of adjacent endothelial neovessels (2) for CD34 (×400). (Color version of figure is available online.)

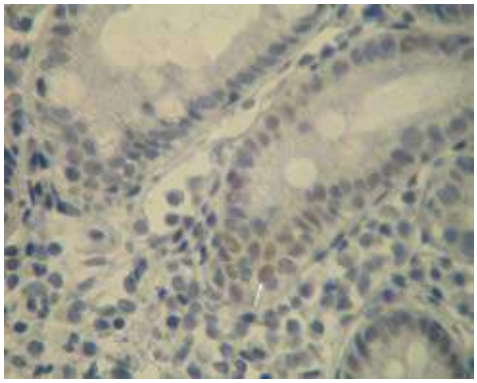

FIG 3. Positive gastric cancer cells (arrow) with nuclear staining for cyclin D1 in another representative tissue region of the same *Helicobacter pylori* positive patient (×400). (Color version of figure is available online.)

DIFFUSE-TYPE GASTRIC CANCER GENETICS AND *H. PYLORI* INFECTION

The data regarding the genetics of diffuse gastric cancer are less complete. Mutations in the E-cadherin gene have been associated with the development of the diffuse-type gastric cancer [2, 40]. In particular, germline mutations in the E-cadherin/CDH1 gene have been recognized in families with an autosomal-dominant inherited predisposition to gastric cancer of the diffuse type [3, 41]. The cumulative lifetime risk of developing gastric malignancy in CDH1 mutation carriers is greater than 70%, and women of these families also have an increased risk of developing breast cancer. Due to this high risk, prophylactic gastrectomies have been performed in several unaffected CDH1 mutation carriers, and despite normal endoscopic evaluations and negative gastric biopsy specimens, pathologic foci of early gastric cancer were observed in all surgical specimens [3]. However, prophylactic gastrectomy results in afflictions of life, and patients with a genetic risk for familial gastric cancer who reject this preventive total gastrectomy must be followed-up intensively by endoscopy and histology every 6 to 12 months [40]. Therefore, a raised awareness among the physician community concerning this syndrome may allow for early detection and prevention of gastric and breast cancers in these high-risk individuals [2, 42]. Of note, apart from intestinal-type cancer, *H. pylori* infection may also be involved in the pathogenesis of diffuse gastric cancer [43].

Further evidence supporting the role for E-cadherin in gastric oncogenesis comes from studies showing that suppression of E-cadherin occurs in 51% of cancers, with a higher percentage found in the diffuse-type cancer. E-cadherin methylation is an early event in gastric

carcinogenesis and is initiated by *H. pylori* infection [44]. Furthermore, E-cadherin under-expression is associated with increased rate of lymph node metastases and decreased survival. Serum soluble E-cadherin is a potential valid prognostic marker for gastric cancer. A high concentration predicts palliative/conservative treatment and T4 invasion. The overall rates of E-cadherin mutations in gastric cancer are low, with the decreased expression of E-cadherin seen in gastric cancer being likely secondary to hypermethylation of the E-cadherin promoter, which occurs in 50% of gastric cancers and 83% of diffuse gastric cancers. In this respect, gastric adenocarcinoma in young patients has a poor prognosis, possesses aggressive histopathological features, exhibits reduced expression of E-cadherin and β-catenin, and demonstrates lower microsatellite instability than tumors in older patients [45].

ATROPHIC GASTRITIS AND *H. PYLORI* INFECTION

Individuals with a family history of gastric cancer are more likely to develop atrophic gastritis (34% *versus* 5%) in the setting of *H. pylori* infection. This genetic predisposition toward the development of atrophic gastritis may reflect different degrees of host immune response to infection. For example, IL-1 cluster polymorphisms have recently been identified as a risk factor for the development of atrophic gastritis and gastric cancer in *H. pylori*-infected patients but not in uninfected patients [2]. Moreover, IL-1 gene cluster polymorphisms are associated with an increased risk in both hypochlorhydria induced by *H. pylori* and gastric cancer [46]. The association with disease may be explained by the biological properties of IL-1β, an important pro-inflammatory cytokine and a powerful inhibitor of gastric acid secretion. Polymorphisms in IL-1β and its endogenous receptor antagonist are associated with risk of *H. pylori*-related gastric cancer [47]. Furthermore, IL-1β polymorphisms enhance not only IL-1β but also IL-8 production in the gastric body and may play an important role in the development of atrophic gastritis. Also, IL-1β polymorphisms are associated with increased risk in gastric cancer not only in whites, but also in patients from the Far East (Japan) [48]. In this respect, long-term acid suppression induced by proton-pump inhibitors (PPIs) in conjunction with *H. pylori* colonization may enhance the development of atrophic gastritis, the well-recognized histological step in the progression to intestinal-type gastric malignancy. Therefore, if patients receive chronic acid suppression therapy, they should be tested for *H. pylori* and treated if positive, due to the potential of PPIs to accelerate atrophy within *H. pylori*-infected mucosa [49]. From another viewpoint, *H. pylori*-infected patients exhibit overexpression mainly of Ki-67 and also of both Bcl-2 and Bax proteins in the upper gastrointestinal tract mucosa, thereby indicating increased proliferation leading to gastric oncogenesis (Table 4) [50]. These last findings, as well as all previously mentioned data on the various mechanisms by which *H. pylori* is involved in gastric carcinogenesis, emphasize the need for *H. pylori* eradication to prevent the development of gastric cancer [51, 52].

CLINICAL RECOMMENDATIONS ON HOW TO FOLLOW PATIENTS WITH *H. PYLORI* INFECTION

Apart from gastroduodenal peptic ulcer disease, eradication of *H. pylori* infection is currently recommended in (1) patients with gastric mucosa-associated lymphoid tissue lymphoma; (2) patients with atrophic gastritis; (3) first degree relatives of patients with gastric intestinal or diffuse type of cancer; (4) patients with unexplained iron-deficiency anemia; (5) patients with gastroesophageal reflux disease needing long-term acid-suppression therapy, due to the potential of PPIs to induce, in presence of the organism, atrophic gastritis, with a subsequent risk in developing gastric cancer; (6) patients with early gastric cancer treated by mucosal resection; and (7) patients with partial gastrectomy for gastric cancer [49, 51, 53]. Complete remission rates, stability of remissions, and frequency of relapse and histological residual disease should be considered.

CONCLUDING REMARKS

In summary, both genetic and environmental risk factors play a significant role in gastric carcinogenesis, leading to either abnormal gene overexpression or inappropriate expression of normal genes, whose products confer the malignant phenotype. Gene mutations could be either inherited (germline mutations) or acquired through various environmental risk factors or failure of intrinsic cellular mechanisms including DNA replication or transcription (somatic mutations). Specifically, the suppression/inactivation of several tumor suppressor genes and the activation of several growth-promoting genes appear to be important in the pathogenesis of gastric cancer. *H. pylori* infection appears to be involved in gastric carcinogenesis through various molecular mechanisms, thereby necessitating eradication of this bacterium.

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Helicobacter pylori infection upregulates endothelial nitric oxide synthase expression and induces angiogenesis in gastric mucosa of dyspeptic patients

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Background **Helicobacter pylori (*H. pylori*) infection induces nitric acid (NO) overproduction through inducible NO synthase (NOS) expression, subsequent DNA damage and enhanced antiapoptosis signal transduction sequence in the human gastric mucosa, whereas its possible effect on endothelial nitric oxide synthase (eNOS) expression has not as yet been investigated. The aim of this study was to evaluate the effect of *H. pylori* infection in the expression of eNOS in gastric mucosa.**

Patients and methods **We prospectively studied 30 nonsmoking dyspeptic patients (12 men, 18 women, mean age 54.26 ± 12.89 years). The diagnosis of *H. pylori* infection was based mainly on histology. The histological grading of *H. pylori* infection was evaluated according to the modified Sydney classification. Histological grading of eNOS expression and microvessel density as estimated by CD34 expression were determined by immunohistochemistry (degree 0–3) and correlated with *H. pylori* infection and histological degree of gastritis.**

Results **Twelve patients were *H. pylori*-positive and 18 patients were *H. pylori*-negative. The two groups were matched for age ($P=0.139$), sex ($P=0.342$) and similar degree of gastritis. Intensity of eNOS and CD34 expression in the corpus and antrum were significantly correlated ($P<0.001$). eNOS expression was correlated with *H. pylori* infection in the mucosa of the body and antrum ($P=0.013$ and 0.037, respectively) but not with gastric inflammation and activity ($P=0.848$ and 0.871, respectively, for the corpus and $P=0.565$ and 0.793, respectively, for the antrum). *H. pylori*-positive patients showed higher expression of CD34-positive blood vessels in the mucosa of the antrum ($P=0.048$). CD34 expression was correlated with gastric inflammation and activity ($P=0.03$ and 0.044, respectively) in the mucosa of the antrum of *H. pylori*-positive patients.**

Conclusion ***H. pylori* infection upregulates eNOS, and induces angiogenesis, contributing to *H. pylori*-associated pathophysiology in gastric mucosa. *Eur J Gastroenterol Hepatol* 20:441–449 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

European Journal of Gastroenterology & Hepatology 2008, **20:**441–449

Keywords: angiogenesis, endothelial NOS, gastric mucosa, *Helicobacter pylori*, nitric oxide

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Received 3 September 2007 **Accepted** 23 November 2007

Introduction

Nitric oxide (NO), a soluble gas first reported as endothelium-derived relaxing factor in 1987, is a short-lived (a matter of seconds) free radical and messenger agent that mediates miscellaneous functions, including vasodilatation, vascular permeability, neurotransmission, regulation of wound healing and nonspecific immune responses to infection, host defense and cytotoxicity. It is synthesized from L-arginine, molecular O_2, and nicotinamide adenine dinucleotide phosphate and other cofactors by the three distinct NO synthase (NOS) isoforms: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). eNOS, a distinctive NOS isoform expressed constitutively by the vascular endothelium (*in vivo* and *in vitro*), seems to be important for the systemic and/or local vascular integrity; its expression by endothelial cells indicates vascular activation. nNOS appears in neurons of the central and peripheral nervous system. Both eNOS and nNOS are expressed under normal conditions referred as constitutive NOS (cNOS). In contrast, iNOS is not constitutively expressed and is induced in response to bacterial endotoxins and by certain cytokines including tumor necrosis factor-α and lipopolysaccharides (LPS), thereby causing NO release [1].

The three enzymatic sources of NO, eNOS, nNOS and iNOS, have been characterized in the gastrointestinal tract. The protective properties of the NO derived from

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cNOS have been well recognized, whereas the role assigned to iNOS is less clear [2]. Specifically, NO plays a crucial role in regulating the diverse mucosal functions and in modulating mucosal integrity of the gastrointestinal tract. Constitutively produced NO is believed to be an essential component of mucosal defense mechanisms, mainly because it increases mucosal blood flow. Furthermore, NO produced by cNOS, is implicated in maintaining the gastric epithelial intercellular barrier integrity and in regulating the gastric mucosa secretory processes. NO protects the gastric mucosa by increasing mucus secretion, and downregulating gastric acid secretion. Moreover, NO interacts with neuropeptides and prostaglandins to maintain mucosal integrity in basal conditions [3]. Repression of NO release by NO inhibitors exaggerate gastric lesions induced by necrotizing agents including ethanol and this effect is competitively antagonized by L-arginine [4]. Despite the fact that most of the evidence suggests a beneficial role of NO to the gastric mucosa, it has been shown that NO exerts a dual role, as in large quantities owing to excess production by iNOS, NO exhibits cytotoxic effects on the vascular system and leads to hemorrhagic injury [5,6]. As iNOS is expressed in the stomach in response to LPS [7], it is thought that NO/iNOS system also plays a role in the mucosal break pathophysiology. It seems that a low level of expression of iNOS reflects a positive host defense response to challenge, whereas exaggerated or uncontrolled expression of iNOS becomes detrimental [2]. Local intra-arterial infusions of high dose of NO causes extensive damage to the stomach [8]. The mechanism of this toxicity involves the superoxide radical that, combined with NO, results in the highly cytotoxic peroxynitrite formation; topical application of peroxynitrite in rat gastric mucosa induces tissue inflammation [9,10].

Helicobacter pylori (*H. pylori*) gastric infection induces an active immune response including stimulation of iNOS expression. Although NO can kill this bacterium, the bacterium persists indefinitely, suggesting that NO production is inadequate [11]. In this respect, chronic *H. pylori* infection induces iNOS expression, subsequent DNA damage and enhanced antiapoptosis signal transduction sequence. This sequence of events supports the hypothesis that oxygen-free radical-mediated damage owing to *H. pylori* plays a crucial role in the development of gastric carcinoma [12]; NO has been related to tumor angiogenesis [13], and polymorphonuclear-derived oxygen-free radicals in *H. pylori*-infected gastric mucosa may contribute to oncogenesis of *H. pylori*-associated gastric carcinoma.

Although eNOS enzymatic source of NO also plays a role in gastrointestinal pathophysiology [6,14], the potential *H. pylori* effect on its expression has not been investigated yet. As upregulation of eNOS expression is an early event in the inflammatory process, induced by a variety of stimuli including vascular endothelial growth factor (VEGF), we evaluated the impact of *H. pylori* infection on eNOS expression in human gastric mucosa, along with the possible correlation to angiogenesis induction as estimated by the microvessel density marker CD34.

Patients and methods

Patients

We prospectively studied 30 consecutive nonsmoking patients (12 men, 18 women, mean age 54.26 ± 12.89 years) referred to our endoscopy unit because of dyspepsia (epigastric pain, bloating, abdominal discomfort, esophageal and/or epigastric burning).

All patients with gastric and/or duodenal lesions diagnosed by endoscopy were excluded from the study. None of the patients had been drinking alcohol or taking corticosteroids, antibiotics, nonsteroidal anti-inflammatory or antisecretory drugs for at least 1 month before the endoscopy. Patients with history of confirmed gastric and/or duodenal ulcer or patients who had received eradication treatment in the past were excluded from the study. Further exclusion criteria included hypertension, renal insufficiency, dyslipidemia under treatment, chronic obstructive pulmonary disease, heart failure, cirrhosis and Crohn's disease.

Each patient gave informed consent to enter the study. The procedures followed in this study were in accordance with the ethical standards of the committee on human experimentation of the institution in which they were done and in accordance with the Declaration of Helsinki.

Methods

Patients reported at 09:00 h after a 12-h fasting. Intravenous sedation with midazolam (2–5 mg) was given, and standard upper gastrointestinal endoscopy was performed with a forward viewing videoscope (Olympus GIF-160; Opto-Electronics Co. Ltd, Tokyo, Japan) to identify evidence of macroscopic abnormalities. Simultaneously, three biopsy specimens were obtained with pinch biopsy forceps (Olympus FB 24K-1) from the antral region within 2 cm of the pyloric ring and three from the corpus (two from the greater and one from the lesser curvature). *H. pylori* detection methods with biopsy urease test and histopathology were described previously [15,16]. In brief, one biopsy specimen from each site was used for rapid urease slide testing of *H. pylori* infection (CLOtest; Delta West, Draper, Utah, USA), and the other two biopsy specimens were placed in 10% formalin and submitted for histological examination.

Biopsy urease test

Each biopsy specimen was placed in a tube containing 0.5 ml of 10% urea in deionized water to which had been

added two drops of 1% phenol red as a pH indicator (CLOtest; Delta West).

Histopathology

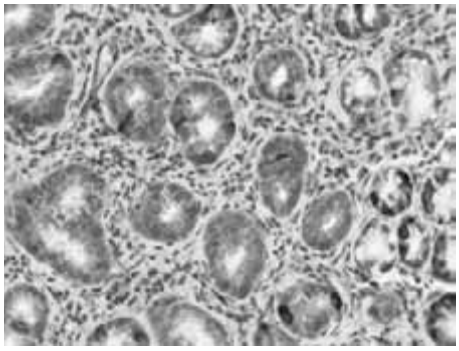
All specimens were stained with hematoxylin and eosin. For detection of *H. pylori* organisms, Crezyl fast violet and/or Giemsa stains were preferred. Moreover, intestinal metaplasia was evaluated with Alcian blue stain. The histological grading of *H. pylori* infection according to the Sydney system [17] included atrophy grade, chronicity, activity and intestinal metaplasia on a scale of 0 (absent) to 3 (high). Two experienced pathologists (V.E. and V.T.) assessed all specimens separately and then they reviewed together histological grading, eNOS and CD34 expressions.

Endothelial nitric oxide synthase immunolocalization

Immunohistochemistry was performed as described previously [18]. In brief, 4-μm-thick serial sections were cut from paraffin blocks, mounted on acid cleaned glass slides, and heated at 55°C for 60 min. Slides were dewaxed and rehydrated; then, the endogenous peroxidase activity was inhibited by incubation with 0.5% H_2O_2 in methanol (10 min at room temperature). To reduce nonspecific background staining, slides were incubated with RTU normal horse serum (10 min at room temperature). To enhance immunostaining, sections were treated with an antigen retrieval solution (10 mmol/l citric acid monohydrate, pH 6.0, adjusted with 2 N NaOH) and were heated three times in a microwave oven at high power for 5 min. Then, sections were washed in buffer solution Tris-buffered saline pH 7.6 (buffer 50 mmol/l Tris, 0.15 mol/l NaCl, pH 7.6 Tris-buffered saline) for 5 min and incubated in the primary antibody, a 1 : 40 dilution of mouse monoclonal antibody against human NOS-3 (NCL-NOS-3, Novocastra Laboratories Ltd, Newcastle, UK). Subsequently, the samples were washed with phosphate-buffered saline for 10 min and incubated with a biotinylated secondary antibody. Sections were rinsed with phosphate-buffered saline and incubated with streptavidin-biotin–peroxidase complex (Dako Corp, Carpenteria, California, USA) for 20 min at room temperature. Sections were developed with chromogen 3,3′-diaminobenzoidine (DAB) and then counterstained with hematoxylin, dehydrated and mounted.

The degree of immunopositivity for eNOS was evaluated semiquantitatively. In random fields from representative areas of the gastric biopsies, the immunoreactive cells were roughly assessed and expressed as a percentage. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm (NOS III) [19,20]. The intensity of immunopositivity was graded as 0 (< 5% cell with positive staining), 1 (5–30%), 2 (30–60%) or 3 (> 60%) (Fig. 1). Two independent approaches were used to confirm antibody specificity: (i) serial dilution of the primary antibody until the immunohistochemical signal disappeared and (ii) the use of nonimmune mouse IgG instead of primary antibodies that failed to reveal relevant staining [18].

Fig. 1



H. pylori gastritis. Endothelial nitric oxide synthase immunostaining in epithelial cells of gastric glands and endothelial cells of mucosal vessels (× 400).

Neovascularization analysis

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

The number of blood vessels per gastric mucosa specimen was calculated by counting the CD34-positive vessels, including monoendothelial vessels, in at least five different fields of representative areas, at × 100 magnification (0.25 × 0.35 mm). Leica IM50 – Image Manager software (Leica Microsystems Ltd, Heerbrugg, Switzerland) applied to a light microscope was used for computer analysis of each sample. Vascularization of gastric mucosa was expressed as mean number of capillaries per biopsy (mean vascular density) [21,22]. The mean vascular density levels were divided into four groups: 0: 0–20 vessels, 1: 20–40 vessels, 3: 40–60 vessels, 4: > 60 vessels (Fig. 2), as described previously [23].

Statistical analysis

The significance of differences was evaluated using the Pearson's χ^2 test (SPSS statistical package, SPSS inc., Chicago, Illinois, USA). Significance was set at $P < 0.05$.

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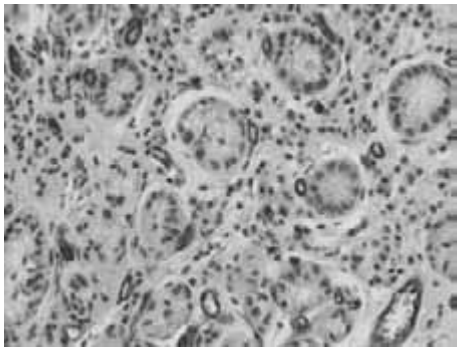
Results

After detailed interview for exclusion criteria, 51 patients were eligible for inclusion to the study at the time of referral for endoscopy. Among them 21 patients were excluded because of gastric or duodenal lesions diagnosed at endoscopy (in one patient a gastric cancer was diagnosed, seven patients presented with duodenal ulcers, nine patients presented with duodenal erosions, eight patients had one or more gastric erosions and in one patient eNOS immunohistochemistry could not be achieved). *H. pylori* infection was detected in 12 of the 30 dyspeptic patients with normal upper gastrointestinal endoscopy who were finally included to the study. The two groups (*H. pylori*-positive vs. *H. pylori*-negative) were matched for age ($P = 0.139$), sex ($P = 0.342$) and degree of gastritis (Table 1), according to the modified Sydney classification [17].

Fig. 2



H. pylori gastritis. High expression of CD34 on blood vessels endothelial cells (× 400).

We observed three main distribution patterns of eNOS immunoreactivity as described previously [24]: along the basolateral surface of mucous cells, in the endothelial cells of mucosal vessels and in cells within gastric glands, located near the basal membrane. Intensity of eNOS and CD34 expression in the gastric corpus and antrum were significantly correlated ($P < 0.001$). Distribution of eNOS and CD34 expression in the two groups are shown in Table 2. eNOS expression was significantly correlated with *H. pylori* infection in the mucosa of the gastric body and antrum ($P = 0.013$ and 0.037, respectively). Gastric inflammation and activity were not correlated with eNOS expression ($P = 0.848$ and 0.871, respectively, for the gastric corpus and $P = 0.565$ and 0.793, respectively, for the gastric antrum).

CD34 expression was not significantly correlated with gastric inflammation and activity in the gastric mucosa of body ($P = 0.358$ and 0.520, respectively) in all patients. In the gastric antrum, in contrast, CD34 expression was significantly correlated with gastric inflammation and activity ($P = 0.047$ and 0.050, respectively). Enhanced angiogenesis was not observed in the gastric corpus mucosa of *H. pylori*-positive patients ($P = 0.168$). The patients with *H. pylori* infection showed significantly higher expression of CD34-positive blood vessels in the gastric mucosa layer of the antrum than those without *H. pylori* infection ($P = 0.048$). Histological findings and CD34 distribution in the antrum of *H. pylori*-positive patients are shown in Table 3.

Table 1 Histological findings

	Gastric corpus			Gastric antrum		
	Hp^+ $N=12$ (%)	Hp^- $N=18$ (%)	*P*	Hp^+ $N=12$ (%)	Hp^- $N=18$ (%)	*P*
Gastritis						
No inflammation	5 (41.6)	6 (33.3)	0.459	5 (41.6)	8 (44.4)	0.866
Low	2 (16.6)	5 (27.7)		2 (16.6)	4 (22.2)	
Moderate	5 (41.6)	5 (27.7)		3 (25.0)	4 (22.2)	
Severe	0 (0.0)	2 (11.1)		2 (16.6)	2 (11.1)	
Gastritis activity						
No activity	8 (66.6)	16 (88.8)	0.128	4 (33.3)	14 (77.7)	0.159
Low	2 (16.6)	0 (0.0)		4 (33.3)	2 (11.1)	
Moderate	2 (16.6)	0 (0.0)		2 (16.6)	0 (0.0)	
Severe	0 (0.0)	2 (11.1)		2 (16.6)	2 (11.1)	
Atrophic gastritis						
Without findings	12 (100)	17 (94.4)	0.592	10 (83.3)	18 (100)	0.162
Low	0 (0.0)	1 (5.5)		2 (16.6)	0 (0.0)	
Moderate	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Severe	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Intestinal metaplasia						
Without findings	10 (83.3)	16 (88.8)	0.331	7 (58.3)	12 (66.6)	0.449
Low	2 (16.6)	1 (5.5)		3 (25)	2 (11.1)	
Moderate	0 (0.0)	1 (5.5)		2 (16.6)	4 (22.2)	
Severe	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	

Hp, *H. pylori*.

Table 2 eNOS and CD34 expression in gastric mucosa of corpus and antrum

	Gastric corpus			Gastric antrum		
	Hp^+ $N=12$ (%)	Hp^- $N=18$ (%)	*P*	Hp^+ $N=12$ (%)	Hp^- $N=18$ (%)	*P*
eNOS						
0 (<5%)	0 (0.0)	0 (0.0)	0.013	0 (0.0)	0 (0.0)	0.037
1 (5–30%)	0 (0.0)	3 (16.6)		0 (0.0)	2 (11)	
2 (30–60%)	2 (16.6)	12 (66.6)		2 (16.6)	12 (66)	
3 (>60%)	10 (83.3)	3 (16.6)		10 (83.3)	4 (22)	
CD34						
0 (0–20)	0 (0.0)	7 (38.8)	0.168	2 (16.6)	10 (55.5)	0.048
1 (20–40)	5 (41.6)	7 (38.8)		3 (25.0)	6 (33.3)	
2 (40–60)	7 (58.3)	4 (22.2)		7 (58.3)	3 (16.6)	
3 (>60)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	

eNOS, endothelial nitric oxide synthase; Hp, *H. pylori*.

Table 3 Histological findings and CD34 distribution in the antrum of *H. pylori*-positive patients ($N=12$)

	0–20	20–40	40–60	>60	*P*
Gastritis					
No activity	2	2	1	0	0.03
Low	0	0	2	0	
Moderate	0	1	2	0	
Severe	0	0	3	0	
Gastritis activity					
No activity	2	2	0	0	0.044
Low	0	1	3	0	
Moderate	0	0	2	0	
Severe	0	0	2	0	
Atrophic gastritis					
Without findings	2	1	7	0	0.233
Low	0	0	2	0	
Moderate	0	0	0	0	
Severe	0	0	0	0	
Intestinal metaplasia					
Without findings	1	3	3	0	0.350
Low	0	1	2	0	
Moderate	0	2	1	0	
Severe	0	0	0	0	

Discussion

The aim of this prospective trial was to investigate the impact of *H. pylori* infection on eNOS expression and angiogenesis induction, estimated by the microvessel density marker CD34, in human gastric mucosa. To the best of our knowledge, the effect of *H. pylori* on eNOS expression and angiogenesis in humans has not been evaluated.

We found that eNOS is overexpressed in the *H. pylori*-infected gastric mucosa irrespectively of the degree of gastric inflammation. eNOS was distributed in all anatomical regions (cardia, corpus and antrum) along the basolateral surface of mucous cells, creating a distinct web of immunoreactivity at the surface and in the pit region of the gastric mucosa. eNOS expression was also detected in endothelial cells of mucosal vessels and in cells near the gastric glands of the body and the antrum. These findings are in accordance, at least partly, with other investigators' findings [24] who described a similar distribution pattern except for their finding of eNOS expression in cells ovoid in shape which contained a round nucleus, located near the basal membrane and did not protrude into the lumen of gastric gland of the oxyntic epithelium. Using double immunostaining, the latter researchers speculated that these cells in close contact with parietal cells were neuroendocrine P cells. Although we did not use double immunostaining, our findings do not support this theory, as we noticed a similar distribution in cells around the gastric glands of the antrum and the body, whereas P cells seem to exist almost solely in the oxyntic part of the human gastric mucosa and rarely in the antral area [25].

Until recently, eNOS has been regarded as a 'static' enzyme that produces a constant amount of NO in both physiological and pathological conditions. This concept has now been abolished and it is clear that it is a far more complicated enzyme than originally anticipated [26,27]. eNOS concentrations are increased in human endothelial cells by VEGF [28] and by a selective estrogen receptor modulator [29]. Exercise training [30] and shear stress also increases by several-fold the expression of mRNA that encodes eNOS [31]. During the inflammatory process, eNOS upregulation is an early event, being the result to several stimuli including shear stress, VEGF and autacoids that are generated locally by tissue injury [27]. NO blocks leukocyte-endothelial adhesion by preventing the synthesis of endothelial cell adhesion molecules such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1 [32] and MAdCAM-1 [33] resulting in inhibition of leukocyte infiltration at the sites of inflammation. In this study, however, eNOS was not correlated with the degree of gastric inflammation suggesting that eNOS triggering happens early in the inflammation cascade, and has an important role in preserving homeostasis in the interaction between the endothelium and inflammatory cells [27].

Apart from eNOS, we found that CD34 is overexpressed in *H. pylori*-infected gastric mucosa, suggesting neoangiogenesis possibly as a result of VEGF induction by *H. pylori* infection [21,22,34–39]; VEGF also induces eNOS

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activity [40]. As, in this trial, the intensity of eNOS and CD34 expression were significantly correlated in patients with *H. pylori* infection, it is reasonable to speculate that the gastric events of this tumorigenic bacterium might involve, at least in part, the effects of eNOS-CD34-VEGF sequence. VEGF is a known angiogenic factor over-expressed in up to 54% of gastric cancers and correlates with the depth of invasion, the staging of gastric carcinoma, an increased risk of lymph node and liver metastases and with disease recurrence [41]. It is important to note that the association between high blood levels of VEGF and poor prognosis in cancer does not depend only on VEGF-induced stimulation of the neovascularization, but also on VEGF-related immuno-suppression (VEGF inhibits dendritic cell maturation and lowers the antitumor cytokine IL-12). Furthermore, IL-8 (induced by *H. pylori* infection) acts as an angiogenic factor for human gastric carcinomas. IL-8 and VEGF (contributing to *H. pylori*-related gastric carcinogenesis) [34,42] may be independent and important prognostic factors in human gastric carcinomas. Moreover, the expression of iNOS and VEGF is closely related to tumor angiogenesis and is involved in the advancement and the lymph node metastases; the microvessel density of the gastric tumor shows correlation with the expression of VEGF (mainly) and eNOS, playing an important role in tumor growth [43].

Specifically, eNOS can be regulated by dynamic sub-cellular targeting, protein–protein interactions and phosphorylation, some of which can be modulated by stimuli in Ca^{2+}-dependent and Ca^{2+}-independent manners [26,44]. A possible interaction between heat shock protein 90 (HSP90) [45–47], Akt (serine/threonine protein kinase B) [26,44,48] and phosphorylation of eNOS has been demonstrated [49,50]. Following stimulation with VEGF, endothelial cells recruit eNOS and Akt to adjacent regions of the same domain of HSP90, facilitating the phosphorylation of eNOS. Thus, a regulatory cascade is involved in the shift of eNOS to a higher level of activation [27] and increased Akt oncogenic signaling pathway is apparent in both stomach cancers and adjacent normal gastric tissues [51]. In-vitro studies confirm that phosphorylation of Akt and its substrates is inducible by epithelial mitogens such as epidermal growth factor, which is implicated in the pathogenesis of *H. pylori* gastritis and gastric cancer [51,52]. Thus, it would appear that VEGF induced eNOS actions may play a role in the gastric events observed in our patients with a potential tumorigenic effect.

Recent data demonstrate that eNOS is present in bone marrow-derived stem cells (BMDCs) and is dynamically expressed during their differentiation into endothelial cells by VEGF *in vitro* [53]. Houghton *et al.* [54] showed that *H. pylori*-induced inflammation in mice caused migration of stem cells originating from bone marrow to the stomach, where they subsequently developed gastric tumors; endothelial cells have also recovered from bone marrow precursors in areas adjacent to dysplasia and neoplasia. In view of these data, we recently conducted a pilot study [55] using tissue sections of biopsies of human gastric cancer in which *H. pylori* bacteria were detected by Cresyl violet staining. Moreover, stem cells and neovessels were detected by immunohistochemical method using a monoclonal antibody anti-CD34; CD34 is a surface glycoprotein expressed on hematopoietic stem cells and is used as an important marker of these cells and neovessels. Therefore, it would be reasonable to speculate that chronic infection of C57BL/6 mice and humans with *H. pylori* induces repopulation of the stomach with BMDCs that may facilitate gastric cancer progression [55]. Extending aforementioned speculation, we can suggest that VEGF-eNOS-CD34 sequence may be correlated with *H. pylori*-induced gastric inflammation and recruitment of BMDCs with their oncogenic consequences. Sustaining this speculation, *H. pylori*-induced CD34 overexpression correlated with inflammation and activity in the gastric antrum mucosa of the patients in this trial. This inflammatory process might promote gastric oncogenesis through induction of atrophic gastritis-intestinal metaplasia, dysplasia, and gastric cancer sequence. Of note, our latter data are in agreement with the findings of Tuccilo *et al.* [22] who also observed more evident expression of VEGF and neoangiogenesis as estimated by CD34 expression in the antrum than in the body of *H. pylori*-infected stomach. Tuccilo *et al.* suggested that predominant antral inflammation could increase expression and local release of gastrin from antral G cells. Gastrin, as an oncogenic growth factor induced by *H. pylori*, is known to stimulate the mutagenic and tumorigenic COX-2 expression and this might in turn lead to increased expression of VEGF [22].

Generally, polypeptide growth factors play a crucial role in the maintenance of gastric mucosal integrity and modulate the balance between proliferation and apoptosis in normal and damaged gastrointestinal mucosa [56,57]. In this respect, VEGF contributes to the restoration of normal mucosal architecture following injury through stimulation of angiogenesis involved in the normal repair process but also in the pathogenesis of inflammatory and ulcerative epithelial lesions as well as malignant tumor growth, and even tumor metastasis [58,59]. Importantly, apart from IL-8 and VEGF, *H. pylori* infection itself induces several other angiogenic factors, such as angiogenin, urokinase-type plasminogen activator and metalloprotease 9. Strowski and colleagues [35] also reported that *H. pylori* stimulates host VEGF gene expression through a mitogen-activated protein kinase pathway. Increase in *H. pylori*-induced angiogenic factors stimulates the recruitment and activation of endothelial

cells in the gastric mucosa, resulting in significant neovascularization of the gastric mucosal layer, which can provide a vulnerable and fertile environment for carcinogenesis. Potent stimulators of angiogenesis related to *H. pylori* infection, *H. pylori Vac*A toxin [36], reactive oxygen species synthesized from neutrophils or macrophages [37], *Cag*A pathogenicity islands [34,38] and lipopolysaccharide have been reported. A wider spectrum of genes induced by *H. pylori* in gastric epithelium was identified by high throughput analysis of cDNA microarray analysis, and most of these genes are responsible for angiogenesis and tumor invasion stimuli, such as a-disintegrin and metalloprotease (ADAM) series, IL-8, VEGF, integrins, VCAM-1, ICAM-1, E-selectin, GRO-α and IL-6 [34,38]. The above-mentioned findings may present a new way of thinking about the pathogenesis of upper gastrointestinal malignancy. More work, however, is needed to elucidate this field.

It is important to note that, our series has relied on histological analysis for the documentation of *H. pylori* infection. Although culture is the theoretic gold standard for detection of the bacterium, it has been shown that there is an excellent correlation with histological identification. Therefore, for most studies, mucosal biopsy and histological examination of the specimen for the presence of *H. pylori* and gastritis is the actual gold standard for diagnosis of *H. pylori* infection [15,16].

One limitation of our study is the rather small number of patients. This is due to strict selection of patients and exclusion of patients with endoscopic lesions. Patients with endoscopic lesions were excluded because mucosal erosions and ulcers present increased angiogenesis during the healing process [60]. Formation of a new microvascular network is essential for the healing of chronic gastroduodenal ulcers. Angiogenesis occurs through a series of sequential steps, which include: (a) degradation of capillary basement membranes by matrix metalloproteinases, (b) endothelial cell migration into the perivascular space and proliferation, (c) formation of microvascular tubes followed by anastomoses, (d) establishment of lumina and basement membranes and ultimately (e) formation of the capillary network [60]. As CD34 is a known neovascularization marker [21–23], we selected patients with normal endoscopy because it would be inappropriate to compare, for example, specimens from ulcer margins or ulcer bed to normal gastric mucosa. It is true that correlation between microscopic and gastroscopic abnormalities is poor [61]; but in this study, histology findings were comparable between two groups.

Finally, a study with an in-vitro culture system could show more directly the effect of *H. pylori* infection in eNOS expression. In-vitro studies, however, have limitations such as that the cells usually used originate from cancer cell cultures and the effects observed could potentially reflect the biology of a tumor cell. Furthermore, our study was designed to evaluate eNOS expression in normally appearing gastric mucosa in correlation not only with *H. pylori* presence but also with inflammation and angiogenesis. Similarly, Tuccillo *et al.* [22], acknowledging the limitations of the in-vitro studies, have conducted an in-vivo study to evaluate the impact of *H. pylori* in VEGF and CD34 expression. Although these investigators had shown that *H. pylori* upregulates the expression of VEGF in human gastric epithelial cells *in vitro* and this was mediated through an EGFR-related and COX-2-related pathways [22,36], the cells used in this study were from an adenocarcinoma. Therefore, to avoid this limitation, we sought to investigate whether angiogenesis is altered *in vivo* in the *H. pylori*-colonized human gastric mucosa along with possible correlation with inflammation and eNOS expression.

In conclusion, our study demonstrated for the first time that eNOS is overexpressed in *H. pylori*-infected gastric mucosa. We also demonstrated CD34 overexpression in the gastric mucosa of the antrum indicating new vessel formation. This event is probably secondary to VEGF induction by *H. pylori* infection as suggested by earlier studies [21,22,34–39]. On this basis, it is also possible that eNOS expression is stimulated as a result to VEGF induction. The identification of *H. pylori*-activated specific signaling pathways leading to the induction of cell cycle and/or angiogenesis mediators may be relevant to the understanding of the mechanism of *H. pylori*-associated gastric carcinogenesis.

Acknowledgement

Conflict of interest: none declared.

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review

Annals of Oncology 19: 1992–1999, 2008
doi:10.1093/annonc/mdn525
Published online 22 July 2008

Primary extranodal lymphomas of stomach: clinical presentation, diagnostic pitfalls and management

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Received 25 January 2008; revised 17 June 2008; accepted 20 June 2008

Gastrointestinal lymphoma is the most common form of extranodal lymphoma, accounting for 30%–40% of cases. The most commonly involved site is the stomach (60%–75% of cases), followed by the small bowel, ileum, cecum, colon and rectum. The most common histological subtypes are diffuse large B-cell lymphoma (DLBCL) and marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue (MALT). *Helicobacter pylori* infection has been implicated in the pathogenesis of MALT gastric lymphoma, but its role in gastric diffuse large B-cell non-Hodgkin's lymphoma (NHL) is controversial. The therapeutic approach for patients with gastric NHL has been revised over the last 10 years. Conservative treatment with anthracycline-based chemotherapy alone or in combination with involved-field radiotherapy has replaced gastrectomy as standard therapy in cases with DLBCL. Additionally, MALT lymphomas are mainly treated with antibiotics alone, which can induce lasting remissions in those cases associated with *H. pylori* infection. Nevertheless, various therapeutic aspects for primary gastric lymphomas are still controversial and several questions remain unanswered. Among others, the role of rituximab, consolidation radiotherapy as well as *H. pylori* eradication in histological aggressive subtypes warrants better clarification.

Key words: diffuse large B-cell lymphomas, extranodal lymphomas, *Helicobacter pylori* infection, mucosa-associated lymphoid tissue, primary gastric lymphomas

introduction

The term primary extranodal non-Hodgkin's lymphoma (PE-NHL) refers to lymphomas which present with disease at any organ or tissue other than lymph nodes or spleen; the symptoms at initial presentation are caused mainly from extranodal involvement and after routine staging procedures, the extranodal involvement remains the clinically dominant site of the disease. PE-NHL comprise ~25%–40% of non-Hodgkin's lymphoma (NHL) and may occur at any organ [1, 2].

Primary non-Hodgkin's lymphoma of the gastrointestinal tract is the most commonly involved extranodal site and represents 10%–15% of all NHL cases and 30%–40% of all extranodal sites [3]. The most commonly involved site is the stomach (60%–75% of cases), followed by the small bowel, ileum, cecum, colon and rectum [4, 5]. All histological categories of nodal lymphomas may also arise in the gastrointestinal (GI), but the main two histological subtypes (>90% of cases) are mucosa-associated lymphoid tissue (MALT) NHL and diffuse large B-cell (DLBC) NHL (Table 1).

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Primary gastric non-Hodgkin's lymphoma (PG-NHL) is localized in the stomach, with or without perigastric and/or abdominal lymph node involvement, and constitutes 20%–30% of all PE-NHL. PG-NHL shows an incidence of 1 per 100 000 of the population in Western countries, but the incidence is progressively increasing. Any histological subtype can arise in the stomach, but the main two histological subtypes (>90% of cases) are MALT NHL and DLBC NHL. *Helicobacter pylori* infection has been implicated in the pathogenesis of MALT PG-NHL [6, 7], but its role in gastric DLBC NHL is controversial [8].

The present review summarizes the clinical presentation, diagnostic work-up and management of patients with primary gastric lymphomas.

diagnosis and staging

Clinical presentation of PG-NHL is not specific and varied, with abdominal pain being the most common symptom followed by dyspepsia, vomiting nausea and anorexia. Weight loss is common, but it is mainly associated with the localization of the disease. Gastric bleeding as presenting symptom occurs in 20%–30% of patients, while gastric occlusion and perforation are less common [4]. Bone marrow involvement, elevated lactate dehydrogenase (LDH) and B symptoms are less

Table 1. Distribution of the main histological types (according to the REAL classification) in the Greek and German study for gastrointestinal non-Hodgkin's lymphoma [4, 5]

Histological type	Greek study (128 patients) frequency (%)	German study (371 patients) frequency (%)
Diffuse large B-cell lymphoma	45	59
With MALT component	9	14
Without MALT component	36	45
MALT lymphoma of the marginal zone	48	38
Follicular lymphoma	2	0.5
Mantle cell lymphoma	1	1
Peripheral T-cell lymphoma	4	1.5

REAL, Revised European-American Lymphoma; MALT, mucosa-associated lymphoid tissue.

common in gastric compared with nodal lymphomas. Endoscopy usually reveals nonspecific gastritis or peptic ulcer with mass lesions being unusual [9]. Occasionally, PG-NHL can present as a multifocal stomach disease with numerous clonally identical foci in macroscopically unaffected tissue [10]. Therefore, gastric mapping of unaffected mucosa is crucially recommended in order to establish diagnosis. Gastric MALT lymphoma is characterized by the presence of lymphoepithelial lesions that are formed by invasion of single glands by aggregates of neoplastic cells with centrocyte morphology [11], in contrast to aggressive lymphoma where lymphoma-infiltrating cells show a centroblastic morphology [12].

Staging work-up for PG-NHL include complete hematological biochemical examinations (including LDH and β2-microglobulin), computerized tomography (CT) of chest, abdomen and pelvis and bone marrow aspiration and biopsy. Upper GI endoscopy and multiple biopsies from stomach, duodenum, gastroesophageal junction and from abnormal-appearing lesions are required. An endoscopic ultrasound should be carried out to determine the depth of invasion and the presence of perigastric nodes. Examination of the pharynx by an otorhinolaryngologist should be carried out to exclude infiltration of Waldeyer ring that is occasionally associated with PG-NHL [13, 14]. In addition to routine histology and immunohistochemistry, cytogenetic studies should be carried out. FISH for the detection of three specific MALT-related translocations is recommended. The pertinent genotypic evaluations should be carried out at the time of diagnosis to guide treatment decisions. Histochemistry (Genta stain or Warthin–Starry stain) and breath test should be carried out to determine the presence of an active *H. pylori* infection. If histology is negative, serology should be undertaken to identify truly negative *H. pylori* gastric MALT NHL which is ∼10% of the cases.

Positron emission tomography (PET) scan bears a documented diagnostic value only for DLBCLs but is controversial for MALT lymphomas, which are frequently reported as PET negative due to their indolent behavior and small tumor volume of disease [15, 16].

The Ann-Arbor classification system [17] is not easily applied to GI tract lymphomas and although alternative staging systems have been proposed, the problem of 'staging' a PG-NHL is controversial even today. The use of different staging systems combined with the variability in staging procedures hamper meaningful comparisons of published series. However, factors that have consistently been associated with poor prognosis in these series are involvement of paraortic (versus local) lymph nodes, serosal penetration and intestinal (versus gastric) origin. An International Workshop in 1994, during the fifth International Conference on Malignant Lymphoma, proposed a modification to Blackledge's system, known as 'Lugano staging' which examines separately local spreading to neighboring anatomic sites [18]. More recently, in 2003, a modified tumor–node–metastasis classification system—the Paris staging system—was proposed in order to describe more efficiently (i) the depth of tumor infiltration, (ii) extent of nodal involvement and (iii) extent of local tissue infiltration by lymphoma [19] (Table 2).

PG MALT lymphomas

Isaacson and Wright [20] first observed in 1983 that primary low-grade gastric B-cell lymphoma and immunoproliferative small intestinal disease share histological characteristics more similar to MALT than those of peripheral lymph nodes. Gastric MALT lymphomas represent the vast majority of the three different types of marginal zone B-cell lymphomas (MZBCLs) according to the Revised European-American Lymphoma (REAL) classification [21]. MALT lymphomas comprise 50% of PG-NHL and are often multifocal. They occur predominantly in individuals >50 years, with a peak in the seventh decade, but cases have been reported in younger patients (third decade or even earlier). In ∼90% of cases, a strong association between chronic *H. pylori* infection and MALT gastric lymphoma has been found [22]. It is accepted that gastric MALT lymphomas arise from MALT acquired as a consequence of *H. pylori* infection and the bacterial infection plays a crucial role in the genesis and development of this tumor [23]. *H. pylori* can be demonstrated in the gastric mucosa of most cases with gastric MALT lymphomas [6]. In addition, epidemiological studies have demonstrated the association between *H. pylori* infection and development of gastric lymphoma [24, 25]. Nevertheless, host immune responses play a less well-defined role in MALT lymphoma formation as indicated by the fact that only a minority of *H. pylori*-infected patients will eventually develop lymphoma.

As with other MZBCL, the cells of PG MALT are typically CD20 positive and express surface and, to a lesser extent, cytoplasmic immunoglobulin (Ig) showing light chain restriction. Most cases express IgM and a few IgA or IgG, but IgD expression is rare. In ∼50% of cases, they aberrantly express CD43. In addition, MALT lymphomas contain moderately high concentrations of CD3+ and CD5+ T cells, but in the majority of cases the lymphoma cells themselves are CD5 negative.

Three translocations, t(11;18)(q21;q21), t(1;14)(p22;q32) and t(14;18)(q32;q21), are specifically associated with MALT lymphomas and the genes involved have been characterized. Although these three translocations involve different genes, they all converge on the activation of the same nuclear factor

Table 2. Comparison of 'Lugano' and 'Paris' staging system for primary GI lymphomas

Lougano staging system [18]	TNM Paris system [19]	Lymphoma extension
Stage I	T1–3 N0 M0	Lymphoma confined to GI tract. Single primary site or noncontiguous lesions.
Stage I1	T1m N0 M0	Confined to mucosa
Stage I2: infiltrating the gastric wall up to the serosa	T1sm N0 M0	Lymphoma infiltrates the submucosa
	T2 N0 M0	Lymphoma infiltrates muscularis propria or subserosa
	T3 N0 M0	Lymphoma penetrates serosa
Stage II	T1–3 N1–2 M0	Lymphoma extending to abdominal lymph nodes
Stage II1	T1–3 N1 M0	Involvement of local (paragastric) lymph nodes
Stage II2	T1–3 N2 M0	Involvement of distant (mesenteric, para-aortic, paracaval, pelvic, inguinal) lymph nodes
Stage IIE	T4 N0–2 M0	Infiltration of adjacent organs or tissues by direct infiltration
Stage IV: extranodal involvement	T1–4 N3 M0	Spread to extraabdominal lymph nodes
or concomitant supradiaphragmatic nodal involvement	T1–4 N0–3 M1	Noncontinuous involvement of separate site in GI tract (e.g. stomach and rectum)
	T1–4 N0–3 M2	Noncontinuous involvement of other organs (e.g. tonsils, parotid gland, ocular adnexa, liver and spleen) or tissues (e.g. peritoneum and pleura)
	T1–4 N0–3 M0–2 B0	Bone marrow not involved
	T1–4 N0–3 M0–2 B1	Lymphomas infiltrates bone marrow
A		Presence of systemic symptoms (fever, night sweats and weight loss >10% BW)
B		Absence of systemic symptoms
X		Bulky mass (lesion of 10 cm or more in the longest diameter)

GI, gastrointestinal; TNM, tumor–node–metastasis; BM, body weight.

kB (NF-kB) oncogenic pathway [23]. Translocation t(11;18), very common in gastric MALT lymphomas as well as MALT lymphomas at other anatomic sites (30% of MALT lymphomas) [26], results in a chimeric fusion between *AP12* and *MALT1* genes [27, 28]. This translocation is not seen in *H. pylori* gastritis and its presence is associated with extension of the disease outside the stomach (regional lymph nodes and/or distal sites) [29]. The t(11;18)(q21;q21) translocation as well as the t(1;14)(p22;q32) can identify cases that will not respond to *H. Pylori* eradication [30].

In *H. Pylori*-associated gastritis and at the early stages of MALT lymphoma, development antigens expressed by *H. pylori* in conjunction with antigen-specific T cells activate the antigen receptor of polyclonal B cells and lead to the interaction of BCL10 and MALT1 proteins and consequently the activation of NF-kB pathway. During the long course of a chronic infection and persistent antigenic stimulation, a subclone may acquire one of the MALT lymphoma-specific translocations and develop a growth advantage. As a result, constitutive activation of NF-kB pathway occurs independently of *H. pylori* infection and the eradication of the bacterium does not reverse the disease process [23]. The scoring system proposed by Wotherspoon et al. [22] reflects this spectrum of proliferation from polyclonal to monoclonal state.

High-grade MALT lymphomas are equivalent to DLBCL in the REAL classification [21] and they have probably transformed from low-grade MALT lymphomas as they share common clone-specific *Ig* heavy chain gene rearrangements with low-grade lesions [31].

treatment of early stage gastric MALT lymphomas

antibiotic therapy

More than 20 studies have shown a high rate of complete remission (CR) of low-grade MALT lymphomas confined to the stomach following eradication of *H. pylori* with antibiotics [32–36]. Therefore, antibiotic treatment is a reasonable initial treatment in low-grade gastric MALT lymphoma provided thorough hematological and endoscopic follow-up takes place. Thorough endoscopic follow-up is recommended because initial diagnostic gastric biopsies do not exclude the coexistence of aggressive lymphoma which requires cytotoxic chemotherapy. Breath test 2 months after treatment to confirm *H. pylori* eradication and repeat endoscopies with biopsies every 6 months for 2 years and then annually to document remission of the lymphoma are recommended. Despite the fact that eradication of *H. pylori* may take place within 1 month of completion of drug therapy, disappearance of lymphoma may take several months and histologic CR may be delayed up to 18 months. When remission occurs, it appears to be stable. If relapse occurs, it is usually associated with *H. pylori* reinfection. Indications also exist that stage I patients with minimal histological lymphoma residuals after *H. pylori* eradication show a favorable course when treated only by regular follow-up with endoscopies and multiple biopsies without administration of oncological therapy, suggesting the potential role of watch and wait strategy in these patients [37, 38]. In patients with histological CR, lymphoma clone can be detected by PCR analysis of the rearranged *Ig* gene on postremission gastric

biopsies in 50% of the cases. This group should be observed closely, whereas long-term negative PCR may indicate cure of the disease [39].

therapy of cases refractory to antibiotics or *H. Pylori* negative

There are no treatment guidelines for the management of patients who show unresponsiveness to antibiotics or for the subset of *H. pylori*-negative cases. This latter group of patients usually does not respond to antibiotics. A choice can be made between conventional therapeutic approaches.

Radiation therapy (RT) alone is a reasonable treatment option in patients with early-stage (stages I and II) gastric MALT lymphomas refractory to antibiotics. Two small prospective series have shown a 100% complete response rate following RT with a median dose of 30 Gy. The first study by Yahalom [40] from Memorial Sloan-Kettering Cancer Center demonstrated only one treatment failure at a median follow-up of 18 months, whereas the one from Schechter et al. [41] showed no treatment failure at a median follow-up of 27 months. Additionally, Tsang et al. [42] reported on 85 patients with MALT lymphoma (17 patients with gastric MALT NHL) receiving RT alone that up to 90% of patients attained a CR with excellent 5-year progression free and overall survival (OS) rates of 98% and 77%, respectively. With the recent evolvement of CT radiotherapy planning, advanced techniques such as three-dimensional conformal radiotherapy and intensity modulated radiotherapy have facilitated the determination of the clinical target volume, thereby reducing the toxicity that is related to the irradiation of normal gastric mucosa and of nearby organs (especially the left kidney). However, side-effects of RT are encountered, most frequently anorexia, nausea and vomiting. Although with the standard dose (30–35 Gy), no delayed toxicity (such as peptic ulcers or GI haemorrhaging) has been reported; the long-term effects of RT on the structure and function of the gastric mucosa remain to be clarified.

There is no consensus regarding the role of adjuvant chemotherapy after antibiotic treatment. The role of chemotherapeutic agents such as alkylating agents, nucleoside analogues or combination chemotherapy for gastric MALT lymphomas refractory to antibiotics has been tested, but only limited data especially on untreated patients with localized disease exist to date. Recently, Nakamura et al. [43] reported CR rates of 89% after oral monotherapy with cyclophosphamide 100 mg/day on patients with gastric MALT NHL, refractory to antibiotic therapy. In this study, the results were comparable to the results achieved after RT; hence, oral monotherapy with cyclophosphamide might also be a suitable second-line therapeutic option after failure of *H. pylori* eradication therapy. The role of the translocation t(11;18) for the prediction of response to chemotherapy is yet under investigation. Recent data support that for oral alkylating agents such as chlorambucil or cyclophhosphamide, the presence of this translocation in gastric MALT NHL is predictive of resistance [44]. CR rates after 1 and 8 years were 42% and 8% for t(11;18)-positive and 89% for t(11;18)-negative patients, respectively ($P = 0.0003$, 8 years). Hence, oral alkylating agents might only be administered in patients without the translocation t(11;18). Nucleoside analogue named cladribine or 2-chlorodeoxyadenosine has been tested in a phase II study in patients with gastric ($n = 19$) and no-gastric MALT NHL at any stage [45]. Patients had to be chemotherapy naive, not responding to *H. pylori* eradication therapy in case of gastric NHL or suffering from relapse after RT. 2-Chlorodeoxyadenosine was administered at a dose of 0.12 mg/kg body weight by i.v. infusion over 2 h on days 1–5 and was repeated every 4 weeks. All patients responded to treatment after a median number of four cycles, and 84% achieved CR including all patients with gastric NHL. Three patients with gastric NHL have relapsed locally after 13, 18 and 22 months and were salvaged with RT. Grade 3 or 4 toxicity World Health Organisation (WHO) is observed in 38% of patients including mainly leukocytopenia, a herpes zoster in one patient and cardiac toxicity in another. In addition, Streubel et al. [46] have shown that the presence of the translocation t(11;18) does not adversely affect the response to 2-chlorodeoxyadenosine chemotherapy. Therefore, 2-chlorodeoxyadenosine can be considered as an effective and relatively safe drug and seems to be a good therapeutic option for patients with gastric MALT NHL being *H. pylori* negative or unresponsive to eradication therapy.

The efficacy of rituximab (monoclonal anti-CD20 antibody) in patients with gastric MALT NHL has not been extensively evaluated. Martinelli et al. [47] reported on 27 patients with gastric MALT NHL, refractory or not eligible for antibiotic therapy, who were treated with rituximab monotherapy at doses of 375 mg/m^2 once weekly for 4 weeks. Forty-six percent of patients had a pathological and clinical CR and 31% had a partial response (PR). With a median follow-up of 33 months, only two patients relapsed whereas there was no association between t(11;18) (q21;21) translocation by FISH and response to treatment. Nevertheless, extrapolating data from randomized studies showing survival advantage in patients with low-grade NHL when rituximab is added to the treatment, we can assume that rituximab is reasonable therapeutic option in patients with gastric MALT NHL refractory to the first-line treatment or in *H. pylori*-negative patients.

In the past, gastrectomy was the treatment of choice in patients with PG-NHL. However, the high morbidity rates associated with this procedure led to attempts to preserve the organ using radiation and combination chemotherapy approaches. Aviles et al. [48] reported the results of a three-arm randomized trial in patients with gastric MALT lymphomas treated in Mexico. They randomized 241 patients to surgery (total gastrectomy) alone versus radiation (30 Gy to the entire abdomen, increased to 40 Gy for the upper abdomen only) alone or chemotherapy [three cycles of combination chemotherapy with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP)-21 followed by four cycles COP-14] alone with median follow-up of 7.5 years. All patients in the three arms achieved CR. Event free survival (EFS) was significantly inferior in radiation (52%) and surgery (52%) arms compared with the chemotherapy arm (87%) ($P < 0.01$). The 5-year OS tended to be superior in the chemotherapy-treated group (87%) versus the surgery (80%) and RT (75%) arms but this did not reach significance ($P = 0.4$) probably due to the lack of power. The authors concluded that chemotherapy

is an effective and well-tolerated treatment for patients with gastric MALT lymphoma. Although this study investigated the role of surgery versus radiation versus chemotherapy as primary treatment of gastric MALT lymphoma, it provides some evidence that combination chemotherapy is more efficacious and durable than radiation in patients with gastric MALT lymphoma who have failed *H. pylori* eradication or for the subset of *H. pylori*-negative cases. In addition, in the German Multicenter Study Group trial, patients with low-grade gastric lymphomas who were treated with surgery and radiation or radiation and COP regimen (six cycles) had equal rates of EFS and 5-year OS (83% and 80%, respectively) [49]. Taken together, these results indicate that organ preservation with chemotherapy combined with radiation can yield equal results to surgery plus radiation in this group of patients.

The addition of rituximab to anthracycline-based combination chemotherapy has not been extensively tested in patients with relapsed gastric MALT lymphomas. In a small retrospective study by Raderer et al. [50], 26 patients with relapsed MALT NHL were treated with rituximab plus CHOP or CNOP. Twenty of 26 patients (77%) achieved a CR and six patients a PR. Toxic effects were mainly hematological, with WHO grade leukocytopenia occurring in 20% of patients. With a median follow-up of 19 months (range 10–45), all patients were alive: 22 were in ongoing remission, while four relapsed between 12 and 19 months after treatment. A clinical trial conducted by the International Extranodal Lymphoma Study Group is currently evaluating the activity of the combination of rituximab and chemotherapy in MALT NHL.

On the basis of combination of the published data, we recommend an algorithm for the treatment of low-grade MALT lymphomas of the stomach. *H. pylori*-positive patients with stage I should have an initial treatment of *H. Pylori* eradication. If *H. Pylori* infection is persistent, reeradication should be attempted. Close follow-up with upper endoscopy and biopsies every 3–6 months is recommended. If complete regression of macroscopic disease is not attained after 12 months, patients should receive radiation alone or rituximab or single-agent chemotherapy if RT is contraindicated. These therapies should be instituted more quickly in patients with progressive disease after antibiotic treatment. In patients with macroscopic remission but with minimal lymphoma histological residuals, watchful waiting with regular endoscopies and multiple biopsies (every 3 months) should be considered. *H. pylori*-negative patients, as well as patients with stage II or/and with t(11;18) translocation should receive antibiotic treatment in addition to close follow-up with endoscopy every 3 months. Combined chemotherapy alone or plus radiation should be initiated if no regression is seen. Nevertheless, the issue is open and further studies are needed to determine the optimal therapy of patients with gastric MALT lymphomas refractory to antibiotics.

treatment of advanced stage gastric MALT NHL

Gastric MALT lymphomas rarely present at advanced stage. Similar to other categories of indolent lymphomas, chemotherapy is not curative and asymptomatic patients can be observed without treatment. Indications for therapy include candidacy for a clinical trial, symptoms, GI bleeding, threatened end-organ function, bulky disease, steady progression and patient preference. Chemotherapy (single-agent or combination regimens) is the treatment of choice in most cases [51]. Locoregional RT is utilized in specific cases such as superior vena cava syndrome. If there is evidence of recurrence, endoscopy is recommended. Management of recurrent cases is similar to follicular lymphomas. Platinum analogues have shown promise [52].

practice points in primary gastric MALT lymphomas

- The t(11;18) translocation as well as the translocation t(1;14)(p22;q32) can identify cases that will not respond to *H. Pylori* eradication.
- Antibiotic treatment is a reasonable initial treatment in low-grade gastric MALT lymphoma provided that thorough hematological and endoscopic follow-up takes place.
- Thorough endoscopic follow-up is recommended because initial diagnostic gastric biopsies do not exclude the coexistence of aggressive lymphoma which requires cytotoxic chemotherapy.
- Radiotherapy alone or rituximab or single-agent chemotherapy should be used for the treatment of patients who fail to response to antibiotics or for the subset of *H. pylori*-negative cases.

DLBCL of the stomach

DLBCL of the stomach is an aggressive lymphoma that might arise *de novo* or from MALT lymphoma transformation. This malignancy constitutes 40%–70% of all gastric lymphomas [4, 5]. High-grade lymphomas bearing the same Ig light chain restriction and identical rearranged *Ig* gene with coexistent low-grade MALT lymphoma represent transformed cases [31, 53]. Foci of DLBCL may be seen in MALT lymphomas but the extent of this high-grade component varies from a small proportion of transformed blasts with the indolent MALT lymphoma to a dominant large cell component with only small residual foci of MALT lymphoma. The differentiation of the latter group from *de novo* large cell lymphomas becomes very difficult. DLBCLs with germinal center-like phenotype (bcl6- and CD10 positive and a proportion bcl2 positive) are not confused with transformed MALT lymphomas. Transformed MALT lymphomas are CD10- and bcl2 negative and bcl6 positive [54]. In most cases, however, immunophenotype as well as molecular genetics cannot reliably distinguish transformed MALT from PG-DLBCL. However, the differentiation between transformed MALT and *de novo* PG-DLBCL is not clinically important since the two entities behave similarly [55]. PG-DLBCL occurs more frequently in males, with median age range of occurrence of 50–60 years [4, 5, 56]. Clinical presentation is similar to that of gastric cancer. The majority of patients report epigastric pain (70% of cases) or dyspepsia (30%). Weight loss is observed in 40% of patients,

more frequently as a result of dyspepsia and less often as a B symptom. Bleeding and perforation are rare at the time of diagnosis. No risk factors have been clearly demonstrated in patients with PG-DLBCL. However, there is some evidence that atrophic gastritis, especially in the setting of immunodeficiency, may be a risk factor for this neoplasm [57].

The role of *H. Pylori* infection in PG-DLBCL is controversial. This bacterium is detected in 35% of DLBCL of the stomach, mainly in cases with concomitant MALT areas (65% versus 15%) [58]. This suggests that most DLBCL may arise from long-standing *H. pylori*-associated MALT lymphomas. In contrast to early reports, recent data have supported that *H. pylori* eradication results in durable histological CR in 50%–63% of patients with gastric DLBCL with concomitant MALT areas [8]. These findings suggest that, at least in the initial phase, high-grade transformation is not necessarily associated with the loss of *H. pylori* dependence.

treatment of DLBCL of the stomach

Treatment of choice for DLBCL irrespective of anatomic site of the lesion is rituximab plus anthracycline-based combination chemotherapy: epirubicin, or adriamycin or mitoxantrone combined with cyclophosphamide, vincristine and prednisone (CHOP, CEOP or CNOP regimen). Although the impact of the addition of rituximab to chemotherapy regimens has not been tested in large clinical trials in patients with PG-DLBCL [59], treatment must include rituximab due to its proven therapeutic benefit in DLBCL [60, 61]. Complications of chemotherapy include gastric outlet obstruction and bleeding while gastric perforation is rare. Therefore, irrespective of the role of gastrectomy as primary treatment of patients with DLBCL of the stomach which as explained below remains controversial, the role of surgical consultant remains essential in the management of DLBCL of the stomach.

The role of surgery in the management of PG-DLBCL is controversial. Many previous studies have suggested that gastrectomy, particularly in stages I and II patients, significantly improves survival [62–65]. In addition, complications such as perforation, obstruction and hemorrhage can be prevented or treated with surgery. However, these complications are rare. Several studies have shown that patients undergoing gastrectomy have a better outcome compared with those having incomplete resection or biopsy alone [66–68]. It is unclear, however, whether the improved outcome is related to low tumor burden which allows complete resection, similarly to low LDH, or the surgery itself. Contrary to the aforementioned reports, other studies have shown that the extent of surgery (excision or biopsy) has no impact on outcome of GI lymphomas [3, 69, 70]. The excellent results obtained with the use of combination chemotherapy, sometimes combined with radiation, have challenged the role of gastrectomy in the management of patients with PG-DLBCL [71, 72]. Some retrospective and prospective studies suggested that conservative nonsurgical treatment achieves equal or better results than gastrectomy (reviewed recently by Ferreri and Montalban [73]). In our study, patients treated with surgery plus chemotherapy had similar OS and disease-free survival after 38 months of median follow-up with patients treated with chemotherapy alone [5]. In addition, a small prospective randomized trial comparing patients with PG-DLBCL treated with combination chemotherapy alone or with surgical resection followed by chemotherapy concluded that gastrectomy is unnecessary (10-year survival rates 96% and 91%, respectively) [74]. However, the question is open and further prospective trials are required to determine the optimal management of this disease.

The role of consolidation radiotherapy is debated. In retrospective studies, the addition of RT was associated with a lower local relapse rate compared with chemotherapy alone [75]. In a prospective study, the combination of six cycles of CHOP-14 followed by involved-field RT (40 Gy) has been associated with a survival rate at 42 months of 91% [76]. Further prospective randomized trials are required in order to answer the question about the role of RT in the treatment of PG-DLBCL.

In addition to chemotherapy, *H. pylori* eradication with antibiotic therapy should always be carried out in localized or extensive PG-DLBCL, especially in cases of PG-DLBCL with concomitant low-grade MALT component [77]. Although PG-DLBCL with MALT component appeared to be independent of the *H. Pylori* antigen drive, two recent studies showed that 60% of patients with PG-DLBCL with MALT areas achieved histological CR after *H. Pylori* eradication, which have been maintained after long follow-up [78, 79].

The choice of treatment for patients with relapsed or refractory disease depends on patient's age, performance status, extension of relapse and previous therapies. At present, high-dose therapy followed by autologous stem-cell transplantation is the treatment of choice for patients in whom chemosensitivity to some kind of salvage treatment is still present. However, only young patients with good performance status and without comorbidities are candidates for this therapy. Gastrectomy can be a suitable approach in elderly patients who experience relapse limited to the gastric wall and exhibit clear contraindications to chemotherapy. Finally, new combinations of chemotherapeutic regimens, immunotherapy and radioimmunotherapy should be tested in prospective phase II trials on patients with relapsed or refractory PG-DLBCL.

practice points in PG-DLBCL

- The treatment of choice is combination of rituximab plus chemotherapy with anthracycline-based regimens (CHOP, CEOP and CNOP).
- The role of gastrectomy is limited in localized disease due to the similar effectiveness of organ-preserving chemotherapy treatment, alone or in combination with radiation.
- *H. pylori* eradication with antibiotic therapy should always be carried out in localized or extensive disease, especially in cases with concomitant low-grade MALT component.

conclusions

In conclusion, the therapeutic approach for patients with PG-NHL has been revised over the last 10 years. It is widely

accepted that MALT lymphomas are mainly treated with *H. pylori*-eradicating antibiotics, which can induce lasting remissions in those cases associated with *H. pylori* infection. Conservative treatment with anthracycline-based chemotherapy alone or in combination with involved-field RT has replaced gastrectomy as treatment of choice in patients with DLBCL. Nevertheless, various therapeutic aspects for PG-NHL are still controversial and several questions remain unanswered. Among others, the role of rituximab, consolidation RT as well as *H. pylori* eradication in histological aggressive subtypes warrants further investigation.

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ORIGINAL CONTRIBUTIONS 21

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

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

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

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

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

Am J Gastroenterol 2009; 104:21–25; doi:10.1038/ajg.2008.87

INTRODUCTION

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

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Presented at the 2008 DDW, San Diego, California, USA, 18–21 May 2008
Received 11 March 2008; accepted 14 June 2008

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

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

METHODS

Patients

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

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

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

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

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

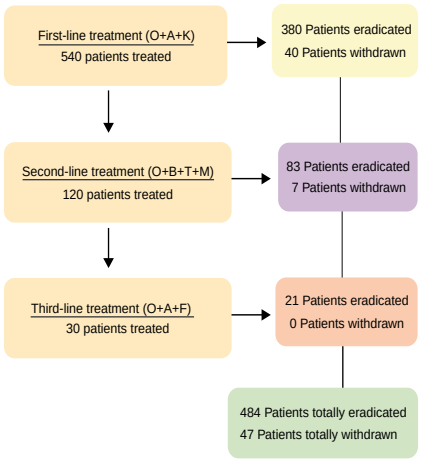
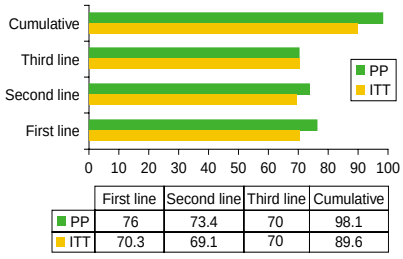


Figure 1. Flow chart summarizing results.

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



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

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

RESULTS

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

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

Table 2. Stratification of results according to indication for treatment

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

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

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

DISCUSSION

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

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

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

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

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

CONFLICT OF INTEREST

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

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

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

Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

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

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

WHAT IS NEW HERE

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

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

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ORIGINAL ARTICLE

Effect of *Helicobacter pylori* Eradication and Antisecretory Maintenance Therapy on Peptic Ulcer Recurrence in Cirrhotic Patients

A Prospective, Cohort 2-year Follow-up Study

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Background: The role of *Helicobacter pylori* eradication to cure peptic ulcer disease in patients with cirrhosis is not clear.

Aim: To investigate the course of peptic ulcer disease in cirrhotics, first after healing with either *H. pylori* eradication or omeprazole therapy and second while on omeprazole maintenance therapy after recurrence.

Methods: Prospective cohort study in a tertiary-care hospital in Greece. Out of 365 consecutive cirrhotic patients who underwent endoscopy, 67 had peptic ulcer and 30 were enrolled. *H. pylori* positive patients received eradication therapy and *H. pylori* negative patients received omeprazole treatment. Follow-up endoscopies were performed at 12 and 24 months or when symptoms recurred. Patients with ulcer recurrence were treated with omeprazole maintenance therapy. The main outcome measurement of the study was peptic ulcer relapse rate during follow-up.

Results: Twenty-eight patients with healed ulcers were followed for up to 2 years. During follow-up, ulcer relapsed in 17 patients (8/18 *H. pylori* positive and 9/10 *H. pylori* negative at study entry, $P = 0.041$), including 2 patients who died from ulcer bleeding. No further ulcer relapse was observed in the remaining 15 patients who received omeprazole maintenance therapy for the rest of follow-up. *H. pylori* negative status ($P = 0.002$) and severity of cirrhosis ($P = 0.015$) at study entry were independently related to shorter peptic ulcer relapse-free time.

Conclusions: *H. pylori* eradication does not protect all cirrhotics from ulcer recurrence and the majority of ulcers recur in *H. pylori* negative patients. Therefore, omeprazole maintenance treatment is mandatory, irrespectively of *H. pylori* status.

Key Words: peptic ulcer recurrence, cirrhosis, *Helicobacter pylori* eradication, maintenance therapy

(*J Clin Gastroenterol* 2008;42:744–749)

Although the prevalence of *Helicobacter pylori* infection in cirrhotic patients with peptic ulcer is similar to that reported in noncirrhotic patients with peptic ulcer from developed countries,[1,2] the prevalence of peptic ulcer disease in patients with cirrhosis is high, ranging from 10% to 49%.[3,4] There is also evidence to suggest greater ulcer recurrence rate compared with the general population.[5] Although different pathophysiologic mechanisms have been implicated in the pathogenesis of peptic ulcer in cirrhosis,[6] it has been reported that the incidence of peptic ulcer is nearly 8 times greater in *H. pylori*-infected cirrhotics compared with uninfected patients with cirrhosis.[1]

It is well established in the general population, that eradication of *H. pylori* reduces dramatically the risk of recurrence of peptic ulcer.[7] However, the role of *H. pylori* eradication in the course of peptic ulcer disease in cirrhotic patients has not been thoroughly investigated. Our study was undertaken to examine the effect of *H. pylori* eradication on ulcer recurrence in patients with cirrhosis and the role of proton pump inhibitor maintenance therapy use in those patients with ulcer recurrence during a 2-year follow-up period.

PATIENTS AND METHODS

The study has been conducted in a single tertiary-care hospital with 800 beds from January 1995 to December 2001in Athens, Greece. In this prospective, follow-up study, we included consecutive cirrhotic patients admitted to our hospital or visiting the outpatients department who had endoscopy to investigate epigastric pain, unexplained anemia, upper gastrointestinal bleeding,

Received for publication October 20, 2006; accepted January 17, 2007.
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or to detect gastroesophageal varices. Enrolled patients fulfilled the following criteria: (1) gastric or duodenal ulcer detected on endoscopy, (2) typical clinical cirrhosis or cirrhosis verified by liver biopsy, laboratory, and imaging studies, (3) consent of patient to participate in the study and to be followed up to 2 years. The exclusion criteria were: (1) previous gastric surgery, (2) aspirin or non-steroidal anti-inflammatory drug (NSAID) use within 2 months before study entry, (3) antisecretory drug (H2 receptor antagonist or proton pump inhibitor) use within 2 months before study entry, (4) history of *H. pylori* eradication therapy, (5) active variceal bleeding or stigmata of recent variceal bleeding at endoscopy, (6) hepatic encephalopathy, (7) hepatocellular carcinoma, (8) other debilitating diseases, that is, malignancy, heart or kidney failure, chronic obstructive pulmonary disease, and stroke.

The study was conducted according to the Declaration of Helsinki for human rights.

Patients' demographic characteristics including sex, age, smoking status, daily ethanol consumption, etiology, and severity of cirrhosis were recorded at study entry. Baseline endoscopy findings including presence, site and size of varices, presence and severity of portal gastropathy, site of peptic ulcer, presence of active bleeding, or presence of stigmata of recent bleeding were also recorded, and also *H. pylori* status.

Etiology of liver cirrhosis was characterized: viral in the presence of chronic viral infection markers (hepatitis B virus, hepatitis D virus, and hepatitis C virus) and alcoholic when there was a history of daily ethanol intake of more than 60 g for men and more than 40 g for women in the absence of chronic viral infection markers. The severity of cirrhosis was classified according to the Child-Pugh criteria.

Gastroduodenoscopies were performed using Olympus GIF-1T100 and GIF-1T140 video-endoscopes. Varices were characterized as esophageal, gastric, or gastroesophageal, small or large in size.[8] Portal gastropathy was characterized as mild or severe.[9] Endoscopically detected mucosal erosive lesion with a crater > 5 mm in diameter, with fibrin-covered base and a distinct border was characterized ulcer—gastric or duodenal. Multiple biopsies were taken from gastric ulcers to exclude malignancy. In case of hemorrhage, bleeding was attributed to the peptic ulcer if the lesion had a visible vessel or fresh or adherent clot or the ulcer was observed to be actively bleeding. Hemostasis was performed with injection of adrenaline solution (1:10000). The presence of *H. pylori* infection was assessed at baseline endoscopy by a rapid urease test (CLO test, Kimperly-Clark, UT) and by histology (modified Giemsa). Two biopsy specimens from the gastric antrum and 2 from the gastric corpus were obtained. One specimen from the antrum and 1 from the corpus were immediately fixed in 10% neutral-buffered formalin for histologic examination and the rest 2 specimens were used for the rapid urease test. Patients were classified *H. pylori* positive if any of the 2 assessments were positive and *H. pylori* negative if both assessments were negative.

Patient Treatment and Follow-Up

Enrolled patients were treated according to their *H. pylori* status: that is, *H. pylori* negative patients received omeprazole 20 mg daily for 4 weeks, whereas *H. pylori* positive patients received a 7-day *H. pylori* eradication therapy with omeprazole 20 mg, amoxicillin 1 g, and clarythromycin 500 mg, all twice daily, followed by omeprazole 20 mg daily for 3 more weeks. Patients were also instructed to avoid using aspirin or NSAIDs during the whole study period.

All patients had a second endoscopy 4 weeks after treatment completion to assess ulcer healing and *H. pylori* status with rapid urease test and histology, as at baseline endoscopy. Patients with healed ulcer and successful *H. pylori* eradication entered the follow-up period.

Patients with unhealed ulcer or persistent *H. pylori* infection were treated as following: (1) *H. pylori* negative patients with persistent peptic ulcer received omeprazole 20 mg daily for 4 more weeks, whereas *H. pylori* positive patients received a 2-week second line quadruple regimen (omeprazole 20 mg twice daily, bismuth subcitrate 125 mg 4 times daily, metronidazole 500 mg thrice daily, and tetracycline hydrochloride 500 mg 4 times daily) followed by omeprazole 20 mg daily for 2 weeks. A new endoscopy was performed 4 weeks later to assess ulcer healing and *H. pylori* status with rapid urease test and histology. Thereafter, patients with complete ulcer healing were included in the follow-up period, whereas those with resistant ulcers were excluded from the study and they were managed individually. (2) Patients with persistent *H. pylori* infection without ulcer received also the second line quadruple regimen. Success of treatment was assessed by urea breath test (Helicobacter Test INFAI, Bochum, Germany) 4 weeks later, and patients entered the follow-up period irrespectively of their final *H. pylori* status.

Clinical evaluation was performed monthly during the follow-up. Patients were asked for symptoms indicating peptic ulcer recurrence and use of NSAIDs or other medication. The first follow-up endoscopy was performed at 12 months and the second at 24 months or earlier if they had epigastric pain or discomfort, unexplained anemia, hematemesis, or melena at any time of follow-up. Patients with ulcer recurrence were treated with omeprazole 20 mg daily maintenance therapy and follow-up continued until the end of the study.

Statistical Analysis

Results are presented as absolute value (%) for qualitative and as median (range) for quantitative data. Qualitative and quantitative data were assessed by nonparametric tests, as appropriate. Life table analysis was performed by the Kaplan-Meier method. The log-rank test was used to examine the variation of recurrence rates of peptic ulcers during the study period. Patients were censored at 24 months if they had not had peptic ulcer relapse by that time. Variables associated with peptic ulcer relapse were entered into a multivariate Cox regression model. A P value (2-sided) of < 0.05 indicated statistical significance for all tests.

RESULTS

During the 7-year study period, 365 consecutive cirrhotic patients underwent endoscopy. Peptic ulcer disease was diagnosed in 67 (18.4%) patients. Thirty-seven patients were excluded from the study because of refusal to consent (N = 10), H2 receptor antagonist use within 2 months before study entry (N = 6), systematic NSAID use within 2 months before endoscopy (N = 4), active variceal bleeding (N = 3), prior anti-*H. pylori* therapy (N = 3), heart failure (N = 2), unwillingness to receive antimicrobial treatment (N = 2), hepatocellular carcinoma (N = 2), uremia (N = 1), chronic obstructive pulmonary disease (N = 1), cerebral vascular accident (N = 1), hepatic encephalopathy (N = 1), gastric cancer (N = 1).

Therefore, 30 patients fulfilling inclusion-exclusion criteria were eligible for enrolment. Eleven of them underwent endoscopy for investigation of varices, 8 for dyspeptic symptoms, 8 for hematemesis and/or melena, and 3 for unexplained anemia.

H. pylori Eradication and Ulcer Healing

There were 19 (63.3%) *H. pylori* positive and 11 (36.7%) negative patients.

Successful *H. pylori* eradication rate was 79% (15/19) and 95% (18/19) after the first line and after the second line eradication treatment, respectively (Table 1). One patient with persistent *H. pylori* infection entered the follow-up period without receiving a third course of *H. pylori* eradication therapy. Side effects of *H. pylori* eradication therapy were mild, including bitterness of taste (N = 7), abdominal fullness (N = 2), headache (N = 1), and diarrhea (N = 1). None of the patients discontinued treatment due to adverse reaction.

Peptic ulcer healed in 18/19 *H. pylori* positive patients. Despite successful eradication treatment, the ulcer in 1 patient did not heal even after the administration of a second course of omeprazole 20 mg daily for 4 weeks. This patient was excluded from the study but he achieved ulcer healing after receiving omeprazole 20 mg twice daily for 4 more weeks.

Out of 11 *H. pylori* negative patients, peptic ulcer healed in 10, after 4 (N = 7) and 8 (N = 3) week's omeprazole 20 mg daily treatment. One patient who did not heal his ulcer after 8 weeks of treatment was excluded from the study. This patient was instructed to receive omeprazole 20 mg twice daily for 4 more weeks, but he did not return for further evaluation.

Ulcer Recurrence During Follow-Up

Twenty-eight patients (24 males, 4 female, aged 48 to 71 y) entered the follow-up period of the study. Their demographic characteristics and baseline endoscopy findings are shown in Table 2. Peptic ulcer was healed in all patients, but 1 patient remained *H. pylori* positive despite 2 courses of eradication treatment.

Median patient follow-up was 24 (3 to 24) months. Peptic ulcer (5 gastric, 12 duodenal) recurred in 17 patients, 13 in the first and 4 in the second year of follow-up. Eight (47.1%) patients presented with abdominal pain, 4 (23.5%) with bleeding, 2 (11.8%) with anemia, whereas peptic ulcer

TABLE 1. Flow Chart of the 30 Patients Included in the Study

H. pylori positive (+) ulcers N = **19**		*H. pylori* negative (-) ulcers N = **11**
Eradication	**Treatment**	**OME**
H. pylori (+)	*H. pylori* (-)	
1	**17 + 1***	**10 + 1***
	Ulcer healed N=28	
	Follow-up period	
1R **4R**	**13**	**8R (2D)** **2**
	12 months	
OME **OME**		**OME**
1 **4** **3R**	**10**	**6** **1** **1R**
	12 months	

*Ulcer not healed, patient excluded from the follow-up period.
D indicates death; N, number of patients; OME, omeprazole 20 mg daily; R, ulcer recurrence.

TABLE 2. Demographic Characteristics and Baseline Endoscopy Findings of the 28 Patients who Entered the Follow-up Period

	N = 28
Sex	
Male	24 (85.7%)
Female	4 (14.3%)
Age (median, range)	60.5, 48-71 y
Etiology of cirrhosis	
Alcoholic	18 (64.3%)
HBV	6 (21.4%)
HCV	4 (14.3%)
Child-Pugh's grade	
A	13 (46.4%)
B	10 (35.7%)
C	5 (17.9%)
Varices	
No	14 (50%)
Esophageal	10 (35.7%)
Gastric	1 (3.6%)
Gastroesophageal	3 (10.7%)
Portal gastropathy	
No	21 (75%)
Mild	5 (17.9%)
Severe	2 (7.1%)
Smoking	
No	15 (53.6%)
Yes	13 (46.4%)
Peptic ulcer site	
Gastric	10 (35.7%)
Duodenal	18 (64.3%)
Bleeding peptic ulcer	
No	20 (71.4%)
Yes	8 (28.6%)
H. pylori status	
Negative	10 (35.7%)
Positive	18 (64.3%)

TABLE 3. Recurrence of Peptic Ulcer According to the Baseline Characteristics of the Patients who Entered the Follow-up Period

	Ulcer Recurrence (N = 17)	No Ulcer Recurrence (N = 11)	*P*
Sex			NS
Male	14 (82.4%)	10 (90.9%)	
Female	3 (17.6%)	1 (9.1%)	
Age (median, range)	65, 48-71 y	57, 48-71 y	0.024
Etiology of cirrhosis			0.041
Alcoholic	8 (47.1%)	10 (90.9%)	
Nonalcoholic	9 (52.9%)	1 (9.1%)	
Child-Pugh's grade			0.043
A	5 (29.4%)	8 (72.7%)	
B	7 (41.2%)	3 (27.3%)	
C	5 (29.4%)	0	
Varices			NS
No	7 (41.2%)	7 (63.6%)	
Yes	10 (58.8%)	4 (36.4%)	
Portal gastropathy			NS
No	13 (76.5%)	8 (72.7%)	
Yes	4 (23.5%)	3 (27.3%)	
Smoking habit			NS
No	9 (52.9%)	6 (54.5%)	
Yes	8 (47.1%)	5 (45.5%)	
Peptic ulcer			NS
Gastric	5 (29.4%)	5 (45.5%)	
Duodenal	12 (70.6%)	6 (54.5%)	
Bleeding peptic ulcer			NS
No	12 (70.6%)	8 (72.7%)	
Yes	5 (29.4%)	3 (27.3%)	
H. pylori status			0.041
Negative	9 (52.9%)	1 (9.1%)	
Positive	8 (47.1%)	10 (90.9%)	

NS indicates no statistical significance.

was detected in 3 (17.6%) asymptomatic patients during scheduled follow-up endoscopy. Two of the patients who recurred with hemorrhage died during the bleeding episode. Ulcer recurred in both patients at 3 months of follow-up and at baseline endoscopy; they both had *H. pylori* negative duodenal ulcer that bled.

All patients with recurrent ulcer were treated with omeprazole 20 mg daily maintenance treatment and continued follow-up. Patients on maintenance treatment did not further recur for the rest 3 to 12 months of follow-up (Table 1).

Table 3 compares baseline characteristics of patients who had or had not ulcer recurrence during the follow-up period. Patient's age ($P = 0.024$), etiology of cirrhosis ($P = 0.041$), and Child-Pugh stage ($P = 0.043$) were significantly related to ulcer recurrence. Peptic ulcer recurred in 8/17 (44%) *H. pylori* positive and in 9/10 (90%) *H. pylori* negative patients during the follow-up period ($P = 0.041$). Moreover, peptic ulcer relapsed in 50% of the *H. pylori* negative patients after 9 [95% confidence interval (CI): 5-13] months mean time of follow-up, whereas the same percentage of *H. pylori* positive patients recurred significantly later, that is, after 18 (95% CI: 14-22) months ($P = 0.032$). At the end of follow-up period, the cumulative hazard for peptic ulcer recurrence was 4 times higher in *H. pylori* negative as compared with *H. pylori* positive patients.

Multivariate Cox regression analysis showed that *H. pylori* positive status and Child-Pugh cirrhosis stage A at study entry were independently related with longer peptic ulcer relapse-free time (odds ratio: 6.1, 95% CI: 1.96-18.87, $P = 0.002$ and odds ratio: 4.2, 95 % CI: 1.32-13.9, $P = 0.015$, respectively).

DISCUSSION

Prevalence of peptic ulcer disease in cirrhotic patients is higher than in the general population.[10] Even more important, ulcer complications, such as acute ulcer bleeding are also more common and could be lethal in cirrhotics.[5,11] Our prospective 2-year follow-up study was designed to investigate the effects of *H. pylori* eradication and proton pump inhibitor maintenance therapy on ulcer recurrence in these patients. In the discussion that follows, *H. pylori* status is always referred to that upon patients' enrolment to the study.

Our data showed that over the study period, ulcer recurrence in *H. pylori* negative cirrhotic patients was

90%. Recurrence rate was 41% in *H. pylori* positive patients after successful eradication and time to recurrences was 9 months later as compared with *H. pylori* negative patients while being without maintenance therapy. No ulcer recurrence was observed while on omeprazole maintenance therapy.

The role of *H. pylori* in peptic ulcer disease in patients with cirrhosis is debatable. A recent publication from Taiwan[12] reported duodenal ulcer recurrence rates 58% after successful *H. pylori* eradication treatment, 44% after failure of *H. pylori* eradication, and 48% in *H. pylori* negative patients during 1 year of follow-up. As these recurrence rates are similar, the authors concluded that eradication of *H. pylori* in patients with cirrhosis cannot effectively prevent ulcer recurrence.[12] However, as it was commented in the accompanying editorial,[13] a beneficial effect of *H. pylori* eradication in a subgroup of patients could not be excluded.

Our study has several methodologic and study population differences from the Taiwan study. First, we included both duodenal and gastric ulcers. Second our study population was about 10 years older (mean age 61 vs. 52 y, respectively), the majority had alcoholic cirrhosis (64% vs. 28%, respectively), cirrhosis was less severe (Child-Pugh stage A: 46% vs. 25%, respectively) and portal gastropathy rate was lower (24% vs. 59%, respectively), as compared with the Lo et al[12] study population. Third, we did not include patients with active variceal bleeding, because these patients need repeated endoscopic interventions for varices eradication that could jeopardize the results of the study due to frequent examinations.[12,13] Fourth, in the Taiwan study, patients were followed for only 1 year after ulcer healing, whereas in our study all recurred ulcers were given omeprazole maintenance therapy and continued follow-up for a second year. Fifth, as it is well known that antisecretory treatment decreases the sensitivity of modalities assessing the success of eradication treatment,[13,14] we clearly defined that *H. pylori* eradication was successful if both rapid urease test and histology were negative 4 weeks after the end of eradication therapy, whereas timing for successful *H. pylori* eradication testing is not clear in the study from Taiwan.[13] Finally, we used multivariate analysis to identify independent factors related to peptic ulcer relapse-free time, whereas investigators from Taiwan used univariate analysis only. Because of these differences, a direct comparison between the studies is not possible. Still, overall ulcer recurrence rate at 1 year was not different, that is, 46% in our patients and 51% in the Taiwan study population.

According to our results, a subgroup of *H. pylori* positive patients benefit from eradication therapy. Peptic ulcer relapsed in 41% of our patients after successful *H. pylori* eradication therapy and eradicated *H. pylori* infection was related with longer peptic ulcer relapse-free time. However, this peptic ulcer relapse rate is much higher as compared with that observed in the general population,[14] suggesting that additional factors could influence ulcer relapse in cirrhosis.

Although controversial, there is evidence to suggest that the incidence and severity of peptic ulcer disease may be greater in patients with decompensate than in those with compensated cirrhosis.[2,3,15] The investigators from Taiwan[12] identified a statistically significant association of duodenal ulcer relapse with the existence of portal gastropathy and the history of variceal bleeding. We also showed that severe cirrhosis (Child-Pugh grades B and C) is related to shorter ulcer relapse-free time. Certainly, future studies are needed to investigate specific mechanisms and factors related to increased risk of ulcer recurrence in cirrhosis.

Because more than 50% of cirrhotic patients will develop recurrent ulcers, it is logical to investigate the role of maintenance antisecretory treatment to prevent ulcer relapse.[13] Di Mario et al[11] have shown that one-fifth of patients with liver cirrhosis on maintenance dosage of H2 receptor antagonists and 30% of patients left without therapy relapsed during a mean period of 24 months. This 21.5% relapse rate was probably because of the well-known tolerance to the antisecretory effects of H2 receptor antagonists, which appears to develop quickly and frequently.[16,17] In our study, ulcer recurrence rate was zero while on standard dose omeprazole maintenance treatment. Furthermore, in 2 of our patients while on no maintenance antisecretory therapy, there was a very early peptic ulcer relapse that resulted to death due to uncontrolled bleeding. Both patients were *H. pylori* negative and they presented with hemorrhagic peptic ulcer at study entry. These findings suggest that the maintenance treatment with a proton pump inhibitor can effectively protect cirrhotic patients from ulcer recurrence and its complications.

Surreptitious use of NSAIDs or aspirin is common and affects the healing and relapse of ulcers.[13,18] We therefore exclude patients with recent use of these medications and during follow-up; we carefully interviewed and instructed our patients to avoid these medications. However, we did not use any laboratory test to monitor NSAIDs and aspirin use, neither we performed gastric outputs studies nor gastrin level measurements to exclude other causes associated with ulcer recurrence, such as occult hypersecretory gastropathy and Zollinger-Ellison syndrome.

A limitation of our study is the relative small size of our sample. During the 7-year study period, out of 365 in and outpatients with cirrhosis, peptic ulcer was detected in 67 (18.4%). This prevalence is slightly higher to the previously reported,[2,3,19] but could be due to the inclusion of inpatients with cirrhosis where the prevalence of peptic ulcer may be as high as 61%.[20] However, only 30 patients fulfilled the inclusion and exclusion criteria, consented to be studied and completed follow-up. There is only 1 publication with 2-year follow-up of patients with cirrhosis and peptic ulcer.[11] In this study, 66% (51/77) of patients were included and completed follow-up. In another study[5] with 1-year follow-up, this percentage was also low (63% or 31/49 patients). These data could be explained by the fact that

patients with liver cirrhosis and peptic ulcer are reluctant to participate in long-term studies requiring endoscopic follow-up.

The results of our study indicate a different scenario regarding peptic ulcer relapse in cirrhosis than that in the general population. Gastric acid secretion is reduced in cirrhosis,[21,22] and it has been suggested that peptic ulcer is more common owing to reduced defensive armament of the gastroduodenal mucosa.[23,24] Furthermore, according to recent publications, *H. pylori* infection is an independent risk factor for peptic ulcer disease in patients with cirrhosis,[1,4,25] although some disagree.[26] It seems that a proportion of *H. pylori* positive peptic ulcer cirrhotic patients may actually benefit from *H. pylori* eradication therapy regarding ulcer relapse, similarly to the general population. In the rest of the patients, gastric acid determines ulcer recurrence.

In conclusion, according to our data, peptic ulcer disease in patients with cirrhosis seems to follow different course after initial healing than peptic ulcer disease in the general population. *H. pylori* negative patients show high ulcer relapse rate and *H. pylori* eradication treatment does not prevent ulcer recurrence in all patients. Therefore, proton pump inhibitor maintenance therapy for all cirrhotic patients with peptic ulcer, irrespectively of their *H. pylori* status, is mandatory to minimize the risk of ulcer recurrence.

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Short Communication

CDH1 gene polymorphisms, smoking, *Helicobacter pylori* infection and the risk of gastric cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST)

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doi:10.1016/j.ejca.2008.02.003

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ARTICLE INFO

Article history:
Received 10 January 2008
Received in revised form
29 January 2008
Accepted 5 February 2008
Available online 14 March 2008

Keywords:
E-Cadherin
CDH1
Gastric cancer
EPIC
Smoking
Helicobacter pylori
Gastric adenocarcinoma

ABSTRACT

Despite declining incidence rates, gastric cancer (GC) is a major cause of death worldwide. E-Cadherin is an adhesion molecule that is thought to be involved in GC. Germline mutations in the E-Cadherin gene (*CDH1*) have been identified in hereditary diffuse GC. Also, a promoter polymorphism at position −160 C/A has been suggested to lead to transcriptional down regulation and has been shown to affect GC risk in some studies. However, very little information exists on the GC risk association of other *CDH1* polymorphisms and it is unclear whether any associations may be different by GC anatomical sites or histological types. Thus, a case–control study (cases = 245/controls = 950) nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort was conducted to assess the GC risk association of eight *CDH1* gene polymorphisms. None of the *CDH1* polymorphisms or haplotypes analysed were associated with GC risk and no differences of effect were observed by *Helicobacter pylori* infection status. However, three *CDH1* polymorphisms in the same haplotype block, including the *CDH1*–160C/A, interacted with smoking to increase GC risk in smokers but not in never smokers. These findings should be confirmed in larger independent studies.

1. Introduction

E-Cadherin (*CDH1*) plays a key role in cell adhesion, which is vital to the normal development and maintenance of cells. Dysfunction of the cell–cell adhesion system triggers neoplastic development. Since *CDH1* is the prime cell adhesion mediator, the gene is thought to serve as a tumour invasion suppressor. Down regulation of *CDH1*, may lead to a loss of *CDH1* mediated cell–cell adhesion, resulting in increased susceptibility to tumour development and subsequent tumour cell invasion and metastasis.[1] In humans, *CDH1* underexpression has been observed in several cancers, including gastric cancer (GC)[2] where it is thought to be stronger in the diffuse than the intestinal sub-type.[3] In fact, *CDH1* inactivating somatic mutations are detected in over 50% of sporadic GCs[4–6] and germline *CDH1* pathogenic mutations are believed to be present in one-third of hereditary diffuse GCs.[7]

Several polymorphisms have been identified in the coding regions of the *CDH1* gene. Of these, the best known is in the −160C/A (promoter region; rs16260), which has shown a 70% reduced level of transcriptional activity of the A allele compared to the C.[8] While two studies on Asian populations show a lower GC risk association for this polymorphism,[9,10] one New Zealand study shows an association for higher GC risk in the diffuse histological sub-type.[11] Other studies in Asian and European populations show no associations.[12–16] Very little information exists on the GC risk association of other *CDH1* polymorphisms.

A case–control study was conducted nested within the European Prospective investigation into Cancer and Nutrition (EPIC-EurGast) to assess the GC risk association of the *CDH1*–160C/A (rs16260) polymorphism and 7 other *CDH1* haplotype-tagging polymorphisms (htSNPs), with the consideration of potential differences by GC anatomical sub-sites, histological sub-type, *Helicobacter pylori* (Hp) infection status and smoking status.

2. Materials and methods

2.1. Subjects

The EPIC-EurGast study was established in order to elucidate the individual and joint effects of dietary/environmental factors, Hp infection and genetic polymorphisms that are putatively involved in GC aetiology in European populations. The study is part of the prospective EPIC study which is detailed elsewhere.[17,18] Cases were gastric adenocarcinomas newly diagnosed during the follow-up period. Gastric lymphomas,

gastric stump cancers, other gastric non-adenocarcinoma and unspecified cancers of the stomach were excluded. For each case (n = 245), up to four controls (n = 950) were randomly selected amongst cohort members alive and free of cancer at the time of case diagnosis, with blood samples available, and matched by gender, age (±2.5 years), centre and date of blood collection (±45 days). This study was approved by the Ethical Review Boards of IARC and all EPIC centres.

GCs were divided into three groups by anatomical sub-site: (i) tumours originating from the gastric cardia (n cases = 69, n matched controls = 257), combining tumours that reached the gastroesophageal junction, either crossing it or from below (all 16 GEJ cancers) or not, (ii) non-cardial tumours (n cases = 128, n matched controls = 508) grouping cases from other sites in the stomach, and (iii) tumours from unknown/mixed sites (n cases = 48, n matched controls = 185). GCs were also divided by histological sub-type according to the Lauren classification: (i) diffuse (n cases = 93, n matched controls = 370), (ii) intestinal (n cases = 96, n matched controls = 372), and (iii) unknown/mixed (n cases = 56, n matched controls = 208). Laboratory methods for Hp infection status are detailed elsewhere.[19]

2.2. SNP selection/genotyping

The software programme *tagSNPs*[20] was used to select a set of htSNPs in which all common SNPs had an estimated pairwise correlation coefficient (R^2_p) > 0.8 with at least one tagging SNP. For all those SNPs poorly correlated with other SNPs, but efficiently correlated with a haplotype of tagging SNP, (R^2S) > 0.8 was also used. When extensive haplotype diversity was observed, the gene was divided into haplotype blocks and the tagging SNPs were selected for each block separately. A haplotype block was defined as the graphical representation of the pattern of linkage disequilibrium (LD) based on D' and selected blocks such that the common haplotypes in each block accounted for at least 80% of all haplotypes observed using the Haploview program.[21] In total, eight SNPs tagging for three *CDH1* haplotype blocks were selected.

Genotyping was performed by Taqman® methodology in 384-well plates read with the Sequence Detection Software on an ABI-Prism7900 instrument, according to the manufacturer's instructions (Applied Biosystems). Primers and probes were supplied by Applied Biosystems (Assays-by-Design™). Each plate included a negative control (no DNA). Positive controls were duplicated on a separate plate. Failed genotypes were not repeated. Assays in which the genotypes of duplicate samples did not show >95% concordance were discarded and replaced with alternative assays with the same tagging properties.

2.3. Statistical analyses

Hardy–Weinberg equilibrium (HWE) for each polymorphism was tested in controls. The association between each SNP

Table 1 – Baseline characteristics and description of the study population of gastric cancer cases and matched controls

	Gastric cancer	
	Cases n = 245	Matched controls n = 950
Age at recruitment[a]	59.1 ± 7.9	59.4 ± 7.8
Age at diagnosis[a]	62.4 ± 8.3	–
Mean number of years between blood donation and diagnosis[a]	3.2 ± 2.1	–
No. of Hp positive subjects[b]	203	646
No. of Hp negative subjects[b]	40	300
Body mass index[a]	26.2 ± 3.8	26.5 ± 4.2
No. of males	138	528
No. of females	107	422
Smoking status		
No. of never smokers	83	418
No. of ex-smokers	87	325
No. of smokers	73	193
No. with missing smoking status	2	14
Grouping by anatomical sub-site		
Cardia, No. of subjects	69	257
Non-cardia, No. of subjects	128	508
Unknown or mixed sub-site, No. of subjects	48	185
Grouping by histological sub-type		
Diffuse, No. of subjects	93	370
Intestinal, No. of subjects	96	372
Unknown or mixed sub-type, No. of subjects	56	208

a Values are means ± standard deviation.

b No. of subjects with missing information on Hp infection status: GC cases = 2, controls = 4. Distribution of cases/controls by EPIC country: Denmark = 22/74, France = 3/12, Germany = 30/120, Greece = 12/48, Italy = 44/173, Netherlands = 19/76, Spain = 29/113, Sweden = 58/224, United Kingdom = 28/110. Details of smoking duration in ex-smokers and smokers: No. of Ex-smokers, duration of smoking < 10 years = 10/44; No. of ex-smokers, duration of smoking ⩾10 years = 72/263; No. of ex-smokers, missing duration of smoking = 5/17; No. of smokers, <15 cigarettes per day = 28/86; No. of smokers, ⩾15–<25 cigarettes per day = 29/59; No. of smokers, ⩾25 cigarettes per day = 10/19.

Table 2 – Odds ratio (OR) and 95% confidence interval (CI) for the GC risk associations of CDH1 polymorphisms, for all GCs and GCs by anatomical sub-site and histological sub-type

CDH1 polymorphism	Genotype	All gastric cancers		GC anatomical site				GC histological type			
				Cardia		Non-cardia		Intestinal		Diffuse	
		Case/ control (*n*)	OR (95% CI)	Case/ control (*n*)	OR (95% CI)	Case/ control (*n*)	OR (95% CI)	Case/ control (*n*)	OR (95% CI)	Case/ control (*n*)	OR (95% CI)
			Age adjusted		Age adjusted		Age adjusted		Age adjusted		Age adjusted
CDH1 –160C/A	CC	119/451	1.0	30/125	1.0	64/243	1.0	46/175	1.0	49/173	1.0
rs16260	CA	101/408	0.9 (0.7–2.2)	31/107	1.2 (0.7–2.0)	51/222	0.9 (0.6–1.3)	39/167	0.8 (0.5–1.5)	37/158	0.8 (0.5–1.3)
	AA	25/90	1.00 (0.6–1.6)	8/25	1.3 (0.5–3.3)	13/41	1.2 (0.6–2.3)	11/30	1.3 (0.6–2.8)	7/37	0.6 (0.3–1.5)
P trend			0.7		0.5		0.9		0.9		0.2
rs1078621	CC	63/281	1.0	15/78	1.0	34/145	1.0	26/108	1.0	24/108	1.0
	CT	125/465	1.2 (0.9–1.7)	37/133	1.4 (0.7–2.7)	3/247	1.1 (0.7–1.8)	49/189	1.0 (0.6–1.8)	47/175	1.2 (0.7–2.1)
	TT	53/188	1.2 (0.8–1.8)	17/46	1.9 (0.8–4.2)	27/99	1.1 (0.6–2.0)	21/74	1.1 (0.6–2.1)	19/75	1.1 (0.6–2.2)
P trend			0.3		0.1		0.7		0.8		0.7
rs4076177	TT	82/350	1.0	23/106	1.0	44/184	1.0	30/138	1.0	34/133	1.0
	TC	122/444	1.2 (0.9–1.6)	32/109	1.4 (0.7–2.5)	63/247	1.1 (0.7–1.6)	48/188	1.1 (0.7–1.9)	47/162	1.1 (0.7–1.9)
	CC	40/145	1.1 (0.7–1.8)	14/39	1.7 (0.8–3.7)	21/74	1.1 (0.6–2.0)	18/45	1.8 (0.9–3.5)	11/66	0.6 (0.3–1.4)
P trend			0.4		0.2		0.7		0.1		0.4
rs7188750	GG	181/666	1.0	51/167	1.0	93/372	1.0	73/260	1.0	69/251	1.0
	GA	55/263	0.8 (0.6–1.1)	15/84	0.6 (0.3–1.2)	30/127	0.9 (0.6–1.5)	19/106	0.6 (0.4–1.1)	20/109	0.7 (0.4–1.2)
	AA	9/21	1.6 (0.7–3.5)	3/6	1.7 (0.4–6.9)	5/9	2.3 (0.7–7.2)	4/6	2.2 (0.6–7.8)	4/10	1.5 (0.4–4.9)
P trend			0.6		0.4		0.6		0.5		0.5
rs3785076	AA	233/866	1.0	64/231	1.0	123/464	1.0	93/336	1.0	89/331	1.0
	AG	10/71	0.5 (0.3–1.0)	4/21	0.7 (0.2–2.3)	4/37	0.4 (0.1–1.1)	3/33	0.3 (0.1–1.1)	2/29	0.3 (0.1–1.1)
	GG	0/1	–	0/0	–	0/1	–	0/0	–	0/1	–
P trend			–		–		–		–		–
rs2276330	AA	185/709	1.0	51/180	1.0	95/394	1.0	74/282	1.0	69/270	1.0
	AG	52/219	0.9 (0.7–1.3)	15/65	0.9 (0.5–1.6)	29/107	1.1 (0.7–1.9)	20/83	0.9 (0.5–1.7)	18/89	0.8 (0.5–1.4)
	GG	7/12	2.3 (0.9–5.8)	2/4	1.7 (0.3–9.5)	4/5	3.8 (0.9–14.4)	2/3	2.2 (0.4–13.5)	5/5	3.8 (1.1–13.5)
P trend			0.7		0.9		0.2		0.8		0.6
rs7203904	GG	149/560	1.0	41/143	1.0	78/314	1.0	62/220	1.0	53/209	1.0
	GC	79/339	0.9 (0.7–1.2)	24/103	0.9 (0.5–1.5)	40/164	1.00 (0.7–1.5)	28/133	0.8 (0.5–1.2)	33/141	0.9 (0.6–1.5)
	CC	17/47	1.4 (0.8–2.5)	4/11	1.4 (0.4–4.4)	10/26	1.6 (0.7–3.4)	6/15	1.5 (0.5–3.9)	7/20	1.4 (0.5–3.5)
P trend			0.8		0.9		0.5		0.7		0.8
rs2276329	AA	221/834	1.0	61/223	1.0	119/447	1.0	90/322	1.0	83/327	1.0
	AG	23/109	0.8 (0.5–1.3)	8/31	1.0 (0.4–2.3)	9/56	0.6 (0.3–1.2)	6/44	0.5 (0.2–1.2)	9/41	0.8 (0.4–1.8)
	GG	1/6	0.6 (0.1–5.7)	0/1	–	0/5	–	0/4	–	1/2	1.9 (0.2–21.5)
P trend			0.3		–		–		–		0.9

Values are odds ratios with 95% confidence intervals.

and GC risk was assessed by odds ratio (OR) and corresponding 95% confidence interval (95% CI) estimated by logistic regression models conditioned on the matching factors plus additional adjustment for age of subject at blood collection (age-adjusted model), plus further adjustments for smoking status/duration/intensity and Hp infection status (mulitvariate-adjusted model). Effect modification by Hp infection status, gender and smoking status/duration/intensity was assessed by the likelihood ratio test. To assess whether Hp infection status, gender or smoking status (never smoker, former smoker, smoker) modify the association of GC risk with *CDH1* polymorphisms, unconditional logistic regression models were used adjusted for the matching factors plus additional adjustment for age of subject at blood collection.

3. Results

3.1. *CDH1 individual SNP analyses*

Baseline characteristics and description of the study population are shown in Table 1.

The *CDH1*–160C/A (rs16260) polymorphism appears to be in linkage disequilibrium with rs1078621 and rs4076177. Although mutual adjustment of these SNPs for each other was attempted in order to determine independent effects, this did not materially alter the findings and so results for these SNPs are presented without any mutual adjustments. All polymorphisms were in HWE. No statistically significant GC risk associations were noted for any of the *CDH1* polymorphisms (Table 2). In the multivariate adjusted model, further adjustments for smoking status/duration/intensity and Hp infection status made no meaningful differences to any of the findings (results not shown).

For *CDH1*–160C/A (rs16260) and most of the other *CDH1* polymorphisms, no differences of effect were observed by GC anatomical site or histological type (Table 2). For rs2276330, the GG versus the AA genotype was associated with higher GC risk in the non-cardia anatomical site (OR = 3.75, 95% CI = 0.98–14.40) and in the diffuse histological type (OR = 3.82, 95% CI = 1.08–13.50).

For all SNPs, no significant interactions were observed between GC risk and gender or Hp infection status. Sub-group analyses by these variables were not remarkable (results not shown). However, consideration of smoking status showed a significant or borderline interaction for the *CDH1*–160C/A (rs16260) polymorphism ($p = 0.02$). Table 3 shows results for sub-group analyses by smoking status. A significantly higher GC risk was observed in smokers for 3 SNPs in the same haplotype block: *CDH1*–160C/A (rs16260), rs1078621 and rs4076177. No meaningful findings were obtained for any of the other *CDH1* SNPs tested (results not shown).

3.2. *CDH1 haplotype analyses*

Haplotypes were also assessed in the context of GC risk. However, no further significant findings were noted when considering haplotypes apart from those manifesting in the SNP analysis.

4. Discussion

Polymorphic variation in the *CDH1* gene promoter region may modulate E-cadherin expression and hence GC risk. However, to date, the findings for polymorphisms in this gene have been inconsistent.[9–16] In the present study, the *CDH1*–160C/A (rs16260) polymorphism was not associated with GC risk, even in sub-group analyses by GC anatomical site or histological type. These results are in line with some of the other studies that have also considered such sub-group analyses showing overall null associations,[12–14] but in contrast with previous findings.[10]

One reason for this inconsistency may be that Hp infection is thought to be required to promote the inactivation of *CDH1* in individuals with the –160CC genotype.[22] Nevertheless, two studies that considered Hp infection status when looking at *CDH1* polymorphisms in association with GC risk have shown that it did not modulate GC risk associated with the *CDH1*–160C/A (rs16260) polymorphism.[12] In the present study, there was also no interaction between Hp infection status and the *CDH1*–160C/A (rs16260) polymorphism.

Table 3 – OR and 95% CI for GC risk associations of selected CDH1 polymorphisms by smoking status

CDH1 polymorphism		All gastric cancers						
	Genotype	Case/ control (*n*)	Never smoker OR (95% CI)	Case/ control (*n*)	Former smoker OR (95% CI)	Case/ control (*n*)	Smoker OR (95% CI)	P-value for interaction
CDH1–160C/A	CC	44/186	1.0	44/161	1.0	29/99	1.0	0.02
rs16260	CA	34/188	0.8 (0.5–1.3)	36/135	1.0 (0.6–1.7)	31/78	1.6 (0.8–3.0)	
	AA	5/44	0.4 (0.2–1.2)	7/29	1.0 (0.4–2.4)	13/16	3.9 (1.6–10.1)	
P trend			0.1		1.0		0.01	
rs1078621	CC	24/129	1.0	26/93	1.0	11/59	1.0	0.25
	CT	42/204	1.1 (0.6–2.00)	43/174	1.0 (0.6–1.7)	40/87	2.9 (1.3–6.4)	
	TT	16/84	1.0 (0.5–2.1)	16/56	1.1 (0.5–2.3)	21/47	2.9 (1.2–6.9)	
P trend			0.9		0.9		0.02	
rs4076177	TT	31/138	1.0	29/129	1.0	20/79	1.0	0.05
	TC	43/206	0.9 (0.5–1.6)	45/145	1.5 (0.9–2.6)	34/83	1.9 (1.0–3.8)	
	CC	9/70	0.5 (0.2–1.2)	12/46	1.3 (0.6–3.0)	19/30	3.1 (1.4–7.0)	
P trend			0.2		0.3		0.01	

Values are odds ratios with 95% confidence intervals.

Interaction with smoking status showed a statistically significant increase in GC risk in smokers for the *CDH*1–160C/A (rs16260), rs1078621 and rs4076177 polymorphisms, all in the same haplotype block. Previously, Lu and colleagues[12] reported that the *CDH*1–160C/A (rs16260) polymorphism is associated with a non-significant increase in non-cardia GC risk in smokers for the CA + AA genotypes versus the CC. It is difficult to speculate exactly how smoking may interact with the *CDH*1 gene, but there are indications from animal models that it may interfere with *CDH*1 expression and function.[23,24] It may even be speculated whether smoking status may explain some of the inconsistencies in results from previous studies. Given that chance is also a possibility for the present observations, these findings should be replicated in other populations using better powered studies.

Haplotype analysis did not show results any different than those presented for the individual SNPs. In general, analysis of haplotypes tests for a potential poly-allelic effect where several linked polymorphisms are thought to modulate cancer risk – but this did not appear to be the case here with the *CDH*1 polymorphisms chosen.

In summary, this study shows no association of any of the *CDH*1 polymorphisms tested with GC risk, particularly the *CDH*1–160C/A (rs16260) polymorphism. No interaction was observed for Hp infection status and no differences of effect were observed in sub-group analyses by GC anatomical site or histological type. Further studies are necessary to replicate these findings and to identify the causal *CDH*1 polymorphisms and their functionality.

Conflict of interest statement

None declared.

Acknowledgements

We thank the members of the pathologist panel for their valuable work: Dr. Roger Stenling, Umea, Sweden; Dr. Johan Offerhaus, Amsterdam The Netherlands; Dr. Vicki Save, Cambridge, United Kingdom, Dr. Julio Torrado, San Sebastian, Spain; Dr. Gabriella Nesi, Firenze, Italy; Dr. U Mahlke, Postdam, Germany; Dr. Hendrik Bläker, Heidelberg; Germany; Dr. Claus Fenger, Denmark and Dr. Dimitrious Roukos, Ioannina, Greece, for his collaboration in the collection of pathological material and Catia Moutinho, Porto, Portugal, for her technical work in the preparation of pathological material. Specific study results of the nested case–control study within EPIC (EUR-GAST) were obtained with financial support from the FP5 of the European Commission (QLG1-CT-2001-01049). The EPIC study was funded by 'Europe Against Cancer' Programme of the European Commission (SANCO); Ligue contre le Cancer (France); Société 3M (France); Mutuelle Générale de l'Education Nationale; Institut National de la Santé et de la Recherche Médicale (INSERM); German Cancer Aid; German Cancer Research Center; German Federal Ministry of Education and Research; Danish Cancer Society; Health Research Fund (FIS) of the Spanish Ministry of Health (RETIC-RD06/0020); the participating regional governments and institutions of Spain; The ISCIII Red de Centro RCESP (C03/09); Cancer Research, UK; Medical Research Council, UK; the Stroke Association, UK; British Heart Foundation; Department of Health, UK; Food Standards Agency, UK; the Wellcome Trust, UK; Greek Ministry of Health; Greek Ministry of Education; Italian Association for Research on Cancer; Italian National Research Council; Compagnia di San Paolo; Dutch Ministry of Public Health, Welfare and Sports; Dutch Ministry of Health; Dutch Prevention Funds; LK Research Funds; Dutch ZON (Zorg Onderzoek Nederland); World Cancer Research Fund (WCRF); Swedish Cancer Society; Swedish Scientific Council; Regional Government of Skane, Sweden; Norwegian Cancer Society.

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A second use of the inhaled insulin test would be to determine if a candidate for inhaled insulin therapy might be prone to develop lung cancer.

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doi:10.1016/j.mehy.2008.05.019

Can Helicobacter pylori infection be proven beneficial to patients with β-thalassaemia major?

Beta-thalassaemia major is characterized by a hereditary defect in the synthesis of β-chains of haemoglobin leading to ineffective erythropoiesis. Conventional management of β-thalassaemia requires regular blood transfusions and systematic use of iron-chelation agents. Inexorable iron accumulation leads to serious organic dysfunction, mainly in heart, liver and endocrine glands, causing even fatal clinical complications [1]. On the other hand, Helicobacter pylori infection has long been associated with iron deficiency anaemia, even in the absence of overt bleeding [2]. Anaemia is unresponsive to iron treatment, or is responsive but exacerbated when supplementary iron is stopped, whereas it is improved by eradication of the bacteria.

Patients with β-thalassaemia major are in greater risk for infectious diseases due to multiple causative factors and infections constitute the second most common cause of mortality in thalassaemia [3]. In order to determine the prevalence of H. pylori infection among asymptomatic patients with β-thalassaemia, we assessed 40 thalassaemic patients (24 F, 16 M, mean age: 27.2 ± 9.7 years) and 30 sex- and age-matched controls for the presence of anti-Helicobacter pylori antibodies in serum using a commercial qualitative and quantitative immunoassay (Varelisa® H. pylori IgG Antibodies, Phadia®, Upsala, Sweden). No significant difference in the prevalence of H. pylori seropositiveness was observed in patients with β-thalassaemia (6/40, 15%) compared to controls (6/30, 20%). As expected, a significant higher mean age and a higher incidence of splenectomy were observed in thalassaemic patients being seropositive to H. pylori. Surprisingly, serum ferritin concentrations were significantly lower in seropositive to H. pylori patients compared to seronegative ones (969.8 ± 677 μg/L vs 2069.5 ± 1250 μg/L, $p = 0.008$). Iron-chelation regimens and reported compliance to treatment were equivalent in all patients.

In a recent study [4], a prevalence of as high as 68% of H. pylori infection was recorded among thalassaemic patients with recurrent abdominal pain; however, not significantly increased compared to controls (60%) with same symptomatology. In this study, no difference in

serum ferritin concentrations was observed between positive and negative to H. pylori thalassaemic patients. However, we should consider the fact that coexisting pathology, causing recurrent abdominal pain, may affect serum ferritin levels in these patients.

It seems that there is a significant interaction between H. pylori and iron metabolism, not fully elucidated yet. And although it is not difficult to associate H. pylori-induced gastric achlorhydria and gastritis to impaired absorption of food iron and periodic bleeding, respectively, the unresponsiveness of anaemia to iron supplementation and its reversal only after bacterial eradication needs additional thinking. Relatively recent, Barabino, based on microbiological and ferrokinetic evidence, speculated a possible diversion of iron to some extramedullar focus being associated to H. pylori infection [5]. Beutler hypothesized that H. pylori subvert the human iron regulatory mechanism by producing hepsidin mimics in a manner that is useful to the micro-organism and deleterious to the host [6]. However, particular for patients with β-thalassaemia this iron sequestration may be proven beneficial. With respect to the limited numbers of our patients, our preliminary data need additional confirmation and further investigation.

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doi:10.1016/j.mehy.2008.05.005

Induced hypothermia as a new approach to bronchopulmonary dysplasia

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that occurs primarily in preterm newborns. No single factor has been identified as the cause of BPD. Its origin is multifactorial and depends on the nature of the injury, mechanisms of response, or the infant's inability to respond appropriately to the injury process [1]. Respiratory support with mechanical ventilation leads to barotrauma and volutrauma. Barotrauma provokes a complex inflammatory cascade that ultimately leads to chronic lung disease. Mechanical ventilation with high tidal volumes markedly increases edema and cytokine production in the lung [2,3]. Prolonged oxygen therapy may cause injury to developing lungs. The premature neonate may be more susceptible to free-radical damage because adequate concentrations of antioxidant may be absent at birth [4,5]. Marked inflammation in the lung appears to begin a cascade of destruction and abnormal repair that progress into BPD. The optimal treatment and prevention strategies for BPD have not been definitively established. Here we propose that therapeutic hypothermia, which modulates many of this processes, may represent a promising therapeutic method for BPD.

Hypothermia has multiple positive effects [6]: (a) hypothermia reduces vasogenic edema, hemorrhage, and neutrophil infiltration; (b) release of excitatory neurotransmitters is reduced, limiting intracellular calcium accumulation; and (c) free-radical production is decreased, which protects

nisms underlie PTSD versus PTSD with comorbid MDD.

Another recent study analyzed data from about 7000 members of the Vietnam Era Twin Registry [4]. This study is the first to use a twin design to quantify the degree to which a common genetic vulnerability explains the etiology of the association between PTSD and MDD. The authors found substantial genetic overlap between PTSD and MDD and suggested that genes implicated in the etiology of MDD are strong candidates for PTSD and vice versa. This observation supports the idea that comorbid PTSD and MDD may be a distinct neurobiological condition that can be named ''post-traumatic mood disorder.'' Post-traumatic mood disorder is associated with greater symptom severity and higher risk for suicidal behavior compared to PTSD [1,2]. Traumatic experiences are common, if we consider the preponderance of individuals exposed to sexual or non-sexual assault, natural disasters (e.g. flooding), accidents (e.g. work, motor vehicle), and war [5]. Many individuals exposed to traumas may have post-traumatic mood disorder. Studies of PTSD and PTSD with symptoms of major depression are merited.

doi:10.1016/j.mehy.2008.01.004

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Helicobacter pylori infection might protect from the leukaemic transformation of myelodysplastic syndromes

Dear Editor,

We read with interest the correspondence of Matsukawa et al. reporting improvement of anemia by *Helicobacter pylori* eradication in a patient with myelodysplastic syndrome (MDS) [1] and that of Hong et al. describing how *H. pylori* infection could improve the survival of patients with carcinomas [2].

We investigated 23 patients diagnosed with MDS and elevated serum title of anti-*H. pylori* IgG antibodies, proving existence of *H. pylori* infection. We conducted Helicobacter INFAI test in order to distinguish between old and recent infection. Surprisingly, all 10 MDS patients without leukaemic transformation had active *H. pylori* infection, whereas in all 13 MDS patients with leukaemic evolution, *H. pylori* was absent, despite the exposure to the infection in the past, as proven by the elevated title of IgG antibodies. The correlations above were statistically significant ($\chi^2 < 0.001$). No eradication therapy was given to any of the patients mentioned.

In this regard, a possible mechanism explaining how MDS patients could benefit from *H. pylori* infection is proposed. It is well established that *H. pylori* stimulates the production of tumor necrosis factor (TNF)-α in peripheral blood [3]. Several studies have implicated the high blood levels of TNF-α with activation of caspases and apoptotic death of marrow cells, through increased free radical production leading to oxidative damage [4—6]. On the other hand, increased apoptosis is connected with early stages of MDS, while leukaemic transformation of MDS arises through inhibition of apoptosis [7]. Therefore, when apoptosis of bone marrow cells is maintained in an increased rate by *H. pylori* infection, the leukaemic evolution is delayed.

It is not reported if the MDS patient mentioned by Matsukawa et al. [1] progressed to acute myeloid leukaemia after the eradication of *H. pylori* infection, despite the improvement of his anemia. It is preferable to treat the anemia of MDS by blood transfusions and iron chelation therapy, while the support of the immune system can be partly accomplished through the production of cytokines (interleukins) by *H. pylori* infection as mentioned by Hong et al. [2] and in accordance with our findings. The immune system support is necessary for the battle against the neoplasmatic clone in MDS marrow. We conclude that *H. pylori* infection could protect from or delay the leukaemic transformation of myelodysplastic syndromes by retaining increased apoptotic rates in the bone marrow. The idea of eradicating *H. pylori* infection in such cases is becoming more and more doubtful.

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doi:10.1016/j.mehy.2008.01.002

A revision of the possible dual role of *Helicobacter pylori* in myelodysplastic syndrome

Dear Editor,

I have read with interest the concept by Diamantidis et al. [1] that *Helicobacter pylori* infection could protect from or delay the leukaemic transformation of myelodysplastic syndrome (MDS) by retaining increased apoptotic rates in the bone marrow via stimulation of the production of tumour necrosis factor (TNF)-α in peripheral blood. Although this notion might be correct, it possibly reflects only one side of the coin. Had *H. pylori*'s role been entirely pro-apoptotic, as the authors suggest [1], this would have meant it could also protect against other malignancies. Instead, *H. pylori* is listed as a class I carcinogen according to World Health Organisation, being the leading cause of gastric cancer and B-cell mucosa-associated lymphoid tissue (MALT) lymphoma, and thus its eradication is recommended [2].

Indeed, as Diamantidis et al. also seem to agree, *H. pylori* induces apoptosis of gastric mucosal cells, being responsible for early atrophy and tissue loss [3]. Apart from TNF-α production, *H. pylori* is further considered as an apoptosis-inducing factor by:

(a) activating the Fas antigen apoptotic pathway [3];
(b) generating reactive oxygen species (ROS) and circulating lipid peroxides [4];
(c) activating mitogen-activated protein kinases (MAPKs) extracellular signal-regulated protein kinase (ERK) 1/2, and MAPK/ERK kinase (MEK) 1/2, mediated at the level of the interleukin (IL)-8 promoter [4];
(d) inducing IL-8 production leading to neutrophil migration and activation, ROS generation and oxidative tissue damage [4].

However, as disease progresses, metaplastic and dysplastic glands resistant to apoptosis arise, gradually leading to gastric adenocarcinoma [5]. *H. pylori* has been shown to induce gastrin production, which in turn upregulates the anti-apoptotic proteins Bcl-2 and survivin [5], involved in the pathogenesis of B-cell MALT lymphoma, and possibly MDS. Interestingly, the expression of the anti-apoptotic proteins Bcl-2 and Bcl-x_L is enhanced in leukaemic transformation, possibly reflecting the anti-apoptotic activity of *H. pylori*.

Concluding, it is deduced that although *H. pylori* exerts a degree of pro-apoptotic activity in early MDS stages, its anti-apoptotic properties will more likely prevail in the long run. In this respect, *H. pylori*'s dual role in apoptosis is further supported by Matsukawa et al.'s study [6], who reported improvement in anaemia after *H. pylori* eradication in a patient with MDS. More research is therefore needed to elucidate the role of this bacterium in MDS.

Competing interests

None

References

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doi:10.1016/j.mehy.2008.03.001

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Helicobacter pylori infection as a risk factor for primary open-angle glaucoma

We read with considerable interest the paper by Casson *et al*.[1] concluding that primary open-angle glaucoma (POAG) in Burmese (Australian) population was associated with increased age, intraocular pressure and myopia. The background prevalence of *Helicobacter pylori* (*Hp*) urea breath test positivity in indigenous Australians is 76%; the prevalence in the remote rural community is 91%, compared with 60% in the urban community.[2] In this respect, although degenerative diseases, including POAG, have an increasingly high impact on aged population, their association with *Hp* infection (*Hp-I*) has only recently been addressed.[3,4]

A relationship between POAG and *Hp-I* appears to exist based on the following comparable data: (i) both diseases mainly affect old people in the developed world; (ii) *Hp-I* has been implicated in a variety of extradigestive vascular conditions including functional vascular disorders, hypertension, ischaemic heart disease and ischaemic cerebrovascular disorders, also detected in POAG and other neurodegenerative diseases contributing to their clinical manifestations;[3–5] and (iii) in the nervous system, *Hp-I* is thought to be associated with the development of autoimmune sequelae observed in peripheral neuropathies, Alzheimer's disease (AD) and glaucomatous optic neuropathy (defined as 'ocular' AD).[3–5]

Based on the above-mentioned data, we documented a high prevalence of *Hp-I* in Greek patients with POAG, establishing a significant relationship between *Hp-I* and glaucoma.[3] Of note, *Hp-I* was confirmed by histology, the gold standard for the diagnosis of *Hp-I*. Reports from neighbouring countries also showed a relationship between glaucoma and *Hp* serology.[4] In a subsequent study we documented a beneficial effect of *Hp* eradication upon glaucoma progression,[4] suggesting a possible causal link between *Hp* and POAG. Moreover, we reported an increased *Hp*-specific IgG antibody level in the aqueous humour of glaucoma patients, the titre of which correlated with the degree of vertical cupping, possibly reflecting the severity of glaucomatous damage.[4]

It would thus be interesting to know if Casson *et al*.[1] have considered comparable data in their rural Myanmar participants expected to exhibit a possible high prevalence of *Hp-I*. Such data appear to be important casting light in *Hp-I* which may influence the pathophysiology of POAG by: promoting platelet and platelet-leucocyte aggregation also involved in the pathophysiology of glaucoma;[4] releasing proinflammatory and vasoactive substances, including cytokines (interleukin-1, -6, -8, -10 and -12, tumour necrosis factor [TNF]-α, interferon-γ), eicosanoids (leukotrienes, prostaglandins) and acute phase proteins (fibrinogen, C-reactive protein) involved in the mentioned vascular disorders and glaucoma;[3–5] 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 *Hp* antigens; producing oxidative stress and circulating lipid peroxides; and influencing the apoptotic process, parameters that may also exert their own effects in the induction and/or progression of POAG[4] and other neurodegenerative disorders (Guillain–Barré syndrome, AD, Parkinson's disease) associated with both *Hp-I* and glaucoma.[4] In particular, increased endothelin-1 (a potent constrictor of arterioles and venules), nitric oxide (NO) and inducible nitric oxide synthase (iNOS) levels are associated with *Hp-I*.[4] Endothelin-1-induced vasoconstriction of the anterior optic nerve vessels and NO modulation of vascular tone in the ophthalmic artery may produce glaucomatous damage. Moreover, NO, a rapidly diffusing gas, is a potent neurotoxin that may facilitate the apoptotic death of retinal ganglion cells in glaucomatous optic neuropathy.[4] 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 POAG.[4]

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Cilioretinal artery occlusion associated with Sub-Tenon's regional blockade

The Sub-Tenon's local anaesthetic block has now been associated with most of the commonly described complications ascribed to ophthalmic blocks, including globe perforation, cerebrospinal fluid injection, haemorrhages and total visual loss.[1] The incidence of these complications, however,

applied directly onto the eyelid margin where the bacteria are thought to reside. Indeed, its benefit remains theoretical. In addition, by using topical antibiotics, their side-effect profile is limited to the local ocular surface to which it is applied. Further studies comparing the long-term effect of prophylactic systemic antibiotic treatment verses prophylactic topical antibiotic treatment in a selection of patients who have a strong history of recurrent chalazia or existing meibomianitis would help to address this question.

Furthermore, long-term systemic antibiotic treatment of *P. acnes* for acne vulgaris has been associated with antibiotic resistance. This problem was found to be more prevalent the longer the antibiotic course.[6] Antibiotic resistance is therefore also likely to become an issue with long-term systemic antibiotic therapy for chalazia.

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Helicobacter pylori infection as an environmental familial clustering risk factor for primary open-angle glaucoma

We read with considerable interest the paper by Green *et al.*[1] concluding that, although glaucoma can be found to cluster in families, it is difficult to ascertain if it is due to exposure to similar environmental factors or a common genetic predisposition.

Our data suggest that both chronic primary open-angle glaucoma (POAG) and pseudoexfoliation glaucoma (XFG) appear to share a common environmental link related to *Helicobacter pylori* (*H. pylori*) infection, the background prevalence of which is high in Greece as well as in Australians (particularly in indigenous Australians).[2,3] Indeed, based on the histological analysis of gastric mucosa biopsy for the documentation of *H. pylori* infection, we have documented a high prevalence of *H. pylori* infection in Greek patients with POAG and XFG.[2,3] In addition, we have documented a beneficial effect of *H. pylori* eradication upon glaucoma progression,[3] suggesting a possible causal link between *H. pylori* infection and POAG and XFG. Moreover, we have reported increased *H. pylori* specific IgG antibody level in the aqueous humour of glaucoma patients, the titre of which correlated with the degree of vertical cupping, possibly reflecting the severity of glaucomatous damage.[3]

POAG and XFG are the two commonest forms of glaucoma in Greece. It is documented that these forms of glaucoma have different pathogenesis, clinical course and prognosis. However, our results show a high prevalence of *H. pylori* infection in both types of glaucoma; it is conceivable that both forms of glaucoma may share a common infectious link related to *H. pylori*. This consideration is further suggested by the observation that Eskimos are the only population in which XFG has not been found because they are isolated in an extremely harsh climate in which the infectious agent cannot survive.[4] An infectious pathogenetic mechanism in XFG was implied in an epidemiologic study in Norway, where the prevalence of the condition was significantly more common in spouses.[5]

Of note, several data indicate that *H. pylori* infection can be found clustering in families. For example, a history of peptic ulcer is more common in Parkinsonism associated with glaucoma and familial transmission of chronic infection, plus part of Parkinsonism links *H. pylori* with causality; relative analysis points to a direct role of the infection in the pathogenesis of Parkinsonism. Besides, in families of children with dyspepsia the infection aggregates and is transmitted; the mother and both parents are involved in the transmission to children. In addition, several lines of evidence suggest that the increased cancer risk in first-degree relatives of gastric cancer probands is mostly dependent on *H. pylori* infection clustering; the prophylactic eradication of *H. pylori* infection in the offspring or siblings of gastric cancer patients may be clinically beneficial.

The possibility that glaucoma patients could be more susceptible to infectious disease may be explained by an existence of a common possible genetic factor that predisposes to both *H. pylori* infection and glaucoma. Genetic susceptibility to *H. pylori* infection has been reported in monozygotic twins. The pathophysiology of glaucoma may also include genetic susceptibility, possibly associated with an immunologic disorder that plays an important role in the initiation and/or sustainment of glaucomatous optic neuropathy. However, future research for detection of common genetic alterations causing susceptibility to both conditions, and how exactly *H. pylori* influences the pathogenesis of glaucoma, is required to elucidate this hypothesis. Understanding the various disorders that play a pathogenetic role in the development of POAG or XFG will allow us to intervene at an earlier stage during the development of the disease, thereby potentially reducing glaucoma blindness rather than just treating, as we currently do, elevated intraocular pressure only.

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The pathogenesis of the glaucomas: nature versus nurture

We thank Kountouras and his colleagues[1] for their interest in our paper.[2] The nature versus nurture argument is common to all work investigating the pathogenesis of complex diseases. The degree of familial clustering can be used to provide both an indicator of common genetic predisposition and common environmental exposure. For example, do obese family members share the same genes or merely the same refrigerator? Kountouras *et al.* raise the interesting possibility that familial clustering seen in primary open-angle glaucoma (POAG) could be due to *Helicobacter pylori* (*H. pylori*) infection.[1]

It is certainly not uncommon for various infections to easily spread between family members and as such be ascribed as having a heritable basis. Leprosy, which prior to Hansen's work was thought to be principally heritable in nature, is a classical example of such a misconception.[3] Interestingly, however, further work has revealed that there are likely to be added genetic factors, such as specific human leukocyte antigen system (HLA) genotypes, which also influence an individual's susceptibility to infection.[3] Combined genetic, epigenetic and environmental factors can all result in familial clustering. Just as leprosy had initially been attributed a genetic pathogenesis and was later found to be due to a gram-positive aerobic bacillus, the converse could certainly be true for the glaucomas. Coincidence of a familial disease and a contagious disease within a family is not uncommon and large well-powered studies are required to prove that discrete associations are real.

Kountouras *et al.* suggest that the association with glaucoma is supported by high rates of *H. pylori* infection in Greece and Australia (particularly the indigenous Australian population). The associated risk between POAG and *H. pylori* infection has not been well replicated.[4] It must also be noted that POAG is extremely rare in the indigenous Australian people, despite their relatively high prevalence of pseudoexfoliation sydrome.[5] Further to this, Kountouras *et al.* postulate that *H. pylori* infection is associated with pseudoexfoliation; however this disease has recently been revealed to have a major genetic component.[6] Given that rates of *H. pylori* infection are particularly high in developing countries and have fallen in developed countries,[7] it will be interesting to observe whether a similar trend is paralleled in the incidence of POAG.

There is good evidence for a genetic predisposition to the primary open-angle glaucomas, and aside from steroid induced ocular hypertension there are no clearly identified environmental stressors which predispose to this disease.[8] It is certain that the glaucomas are a heterogeneous group of conditions, which have a poorly understood, complex aetiology. Research into all facets of this disease's pathogenesis – be it genetic, epigenetic or environmental – should be supported.

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LETTER TO THE EDITOR

Greek and Israeli Patterns of *Helicobacter pylori* Infection and Their Association With Glaucoma: Similarities or Diversities?

To the Editor:

We read with considerable interest the paper by Kurtz et al[1] that showed no association between *Helicobacter pylori* infection and the occurrence of glaucoma. Moreover, the possible explanation for the discrepancy of Dr Kurtz et al's[1] findings and those of our studies[2,3] stemming from different *H. pylori* infection occurrence rates between Greece and Israel was attempted to be dispelled by epidemiologic studies that demonstrated that both countries share similar patterns.[4,5] However, this conclusion is, at least partly, not correct. It has now become clear that the prevalence of *H. pylori* infection is decreasing owing to the wide use of eradication practices. Over a 10-year period, Apostolopoulos et al,[4] who conducted one of the epidemiologic studies mentioned by Dr Kurtz, found a significant decrease in *H. pylori* infection in Greece. Likewise, in Tel Aviv, by using the ^{13}C-urea breath test, broadly used as the noninvasive diagnostic test of choice, having a significantly higher diagnostic accuracy than serology particularly in older subjects,[6] Niv et al[7] also found a rather lower prevalence of *H. pylori* infection in 33% of the elderly people studied. Surprisingly, Dr Kurtz reported a much higher prevalence of *H. pylori* infection in his age-matched and ethnicity-matched controls (61.1%). The reason for this tremendous difference in the prevalence of *H. pylori* infection in the control groups between the studies of Dr Kurtz and Dr Niv may be explained by the extremely small number of subjects (n = 36) included in the first study (n = 36 vs. n = 2128, respectively).

More importantly, the authors of the present study observed, again in Tel Aviv, by using ^{14}C-urea breath test, a seasonal variation in the prevalence of *H. pylori* infection.[8] Specifically, they found a significant decrease in *H. pylori* prevalence during the summer (July-September) compared with winter (December-January) (42.3% vs. 60.9%, $P < 0.007$; results from 371 *H. pylori*-positive participants). According to the results in the present study by Dr Kurtz, all their participants might have been recruited during the winter because they found such a high prevalence of *H. pylori* infection. On the other hand, had their subjects been recruited mainly in the summer (42.3%), a statistical difference in *H. pylori* prevalence between their glaucoma patient and controls might have been observed. In any case, the most significant limitation in this study is the very low number of patients enrolled.

From another viewpoint, *H. pylori* positivity in Israel is also associated with ethnic variations [Sephardic (Asian and African origins) versus Ashkenazi (European and American origins)],[9] which should have been taken into consideration to determine the prevalence of *H. pylori* infection in both glaucoma patients and control subjects.

In view of the abovementioned data, the explanation offered by Dr Kurtz for the discrepancy between his and our studies stemming from different *H. pylori* infection occurrence rates between Greece and Israel was certainly not dispelled by some epidemiologic studies that demonstrated that both countries share similar patterns, as different patterns do exist.

With regard to the potential argument that we also used a small number of patients, in our studies *H. pylori* infection was mainly determined by histologic detection of the bacteria in mucosal biopsy specimens, considered to be the actual gold standard for the diagnosis of this infection. It is important to note that the serologic test has limitations because it does not discriminate between current and old infections. Such a distinction is essential because current *H. pylori* infection induces humoral and cellular immune (predominant *H. pylori*-specific T_H1 response with a T_H1-type cytokine production leading to gastric epithelial cell apoptotic damage) responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves, thereby affecting or perpetuating neural tissue damage including glaucomatous optic neuropathy. Moreover, eradication of *H. pylori* infection seems to delay glaucoma progression, particularly at early disease stages. Although it was too difficult to obtain consent of our glaucoma patients to be submitted to an upper gastrointestinal endoscopy, we managed to recruit a number of 41 by using this gold diagnostic standard, and thus we consider this number as rather reliable for our conclusions. Similar results to ours were also reported by a study from neighboring Turkey, which also showed a possible relationship between glaucoma and *H. pylori* infection by using serology.[10]

Moreover, differences might exist particularly in the prevalence of pseudoexfoliation glaucoma between Greek and other populations, with a high incidence in Greece and Scandinavia. It would be of interest to know its incidence in Israel (Sephardic or Ashkenazi people) that might reveal further epidemiologic differences or similarities with the Greek glaucoma patients.

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doi:10.1016/j.mehy.2008.05.008

Helicobacter pylori may hold a variable role in multiple sclerosis based on ethnicity

Dear Editor,

We have read with interest the hypothesis by Pezeshki et al. [1] that, based on the finding that *Helicobacter pylori* infection (*Hp*-I) is a protective factor against conventional multiple sclerosis (MS) in Japanese patients [2], *Hp* nanoparticles could be applied as a targeted therapy in these patients. Although this therapy might be introduced in Asians, it might be not applicable in Caucasians. Using histology, recognised as the practical gold standard for the diagnosis of current *Hp*-I, our series showed a strong association between *Hp*-I and MS in Caucasians [3]. Although the serological test used by Li et al. [2] can establish *Hp* presence, it cannot discriminate between current and old infections. Such a distinction is crucial because current *Hp*-I induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with nerve components, thereby contributing and possibly perpetuating neural tissue damage. Moreover, eradicating *Hp*-I might alter the MS pathophysiology.

Because CD4+ autoreactive T lymphocytes (TLs) activation may play an important role in the early and long-term evolution of MS, as mentioned by Pezeshki et al. [1], it should be noted that *Hp*-associated gastroduodenal pathologies can be regarded as a Th1-driven immunopathological response to a number of *Hp* antigens. Cytolytic TLs infiltrating the gastric mucosa cross-recognise different epitopes of *Hp* proteins and gastric H^+-K^+-ATPase autoantigen (a significant proportion of the CD4+ T cell clones proliferated in response to H^+-K^+-ATPase showing a Th1 profile), and this bacterium may lead to gastric autoimmunity and atrophy via molecular mimicry. A predominant *Hp*-specific Th1 response leads to apoptotic damage of gastric epithelium. The Fas/Fas ligand (FasL) system is involved in *Hp*-induced apoptosis, and TL-mediated cytotoxicity via Fas/Fas-L signalling may contribute to induction of gastric epithelium apoptosis in the context of *Hp*-I. Moreover, *Hp* induces apoptosis through the mitochondrial apoptotic pathway, involving activation of the Bax and Bak proapoptotic proteins, certain caspases, or through inducible nitric oxide, a potent neurotoxin that contributes to apoptotic neuronal cell death [3]. Therefore, the irregular *Hp*-associated cellular immune and apoptotic mechanisms may affect the neurodegenerative process in MS.

Because *Hp* prevalence is significantly lower in gastro-oesophageal reflux disease (GORD) patients from East Asia than in those from Western countries, possibly indicating a protective role against GORD [4], comparable data might also explain either the likely protective role of *Hp* against MS in Asians or its causative role in MS in Caucasians.

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doi:10.1016/j.mehy.2008.05.007

Inhaled insulin for the early detection of lung cancer

Early lung cancer detection may save lives but is controversial [1]. Recently a study appeared documenting improved survival of early lung cancer detected with spiral CT [2].

Biochemical methods of early lung cancer detection have been developed. For example, measurement of an autoantibody response to one or more tumor-associated antigens in an optimized panel assay provides a sensitive and specific blood test to aid the early detection of lung cancer [3].

Early lung cancer can also be detected by obtaining fluid and tissue samples. Respiratory tract material, including sputum or bronchial fluid or other pulmonary tissue or thoracic cells or regional lymph nodes, is assayed with monoclonal antibodies for antigens whose enhanced presence correlates with the development of lung cancer [4].

Surface enhanced laser desorption/ionization (SELDI) mass spectrometry has been used to identify five distinct potential lung cancer serum biomarkers with high sensitivity and specificity [5].

Very small malignant lung tumors might be detected by allowing the subject to inhale powdered insulin, preceded and followed by measurement of circulating hormones, growth factors, and receptor proteins in the blood, particularly those that lung tumors are known to produce:

- Antidiuretic hormone (ADH) is ordinarily produced in the hypothalamus and secreted from the posterior lobe of the pituitary gland. Lung tumors produce ADH. This hormone is involved in the maintenance of the extracellular fluid environment by reducing free water clearance [6].
- Adrenocorticotrophic hormone (ACTH) is the most common ectopic hormone that lung tumors produce. Like ADH, increased serum levels of ACTH in patients with lung cancer are frequent and may be detectable in up to 50% of cases [6].
- Atrial natriuretic peptide is another hormone produced ectopically by lung cancer cells, which affects renal salt and water handling. In individual patients, increased levels of atrial natriuretic peptide may contribute to hyponatremia by causing natriuresis [6].
- Parathyroid hormone (PTH) and parathyroid hormone related protein (PTHRp) are produced by malignant lung tumors [6] and can lead to hypercalcemia, most commonly in squamous cell carcinomas.

Insulin is a growth factor and causes tumor stimulation. Increased serum insulin levels are associated with an increased incidence of prostate cancer, and also a poor prognosis in prostate and breast cancer [7—9]. Moreover, Pfizer Inc. reported an increase in lung cancer among patients who used its discontinued inhaled insulin Exubera. A review of clinical trial data found there were six cases of lung cancer among 4740 patients using Exubera, compared with one of 4292 who did not take Exubera. All cases were in former cigarette smokers [10].

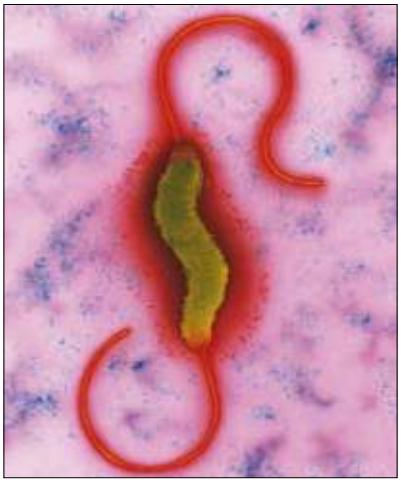
To diagnose lung cancer, inhaled insulin would be used to stimulate lung tumor cells to produce increased blood levels of hormones and other lung cancer markers. Subjects would undergo venepuncture, have blood collected, and receive a single dose or multiple doses of inhaled insulin. After the last insulin dose, blood would be obtained by venepuncture and measurement made of Antidiuretic hormone, Adrenocorticotrophic hormone, Atrial natriuretic peptide, parathyroid hormone, parathyroid hormone related protein, and other lung cancer serum markers. The levels of the hormones and markers would be compared pre and post insulin inhalation. If there had been a significant elevation of one or more hormones or markers after insulin inhalation, as compared to the results from controls without lung cancer, subjects would be sent for further evaluation with spiral CT scanning.

Reflection and Reaction

Guillain-Barré syndrome

We read with interest the Review by van Doorn and co-workers.[1] These authors state that *Campylobacter jejuni* is the most commonly identified infection that precedes Guillain-Barré syndrome (GBS), and they discuss evidence that this infection contributes to the pathogenesis of GBS through molecular mimicry and a cross-reactive immune response. However, the authors did not mention *Helicobacter pylori* infection, a crucial antecedent infection that, like *C jejuni*, seems to be associated with the development of autoimmune sequelae observed in peripheral neuropathies and GBS.[2,3]

We have reported that *H pylori* infection, documented by histology, is more frequent in patients with GBS (12 of 13) than in controls (10 of 20; p=0·02). We found that high serum concentrations of anti-*H pylori* IgG antibodies were closely correlated with a more advanced clinical stage of GBS; furthermore, increased serum antibody concentrations were associated with involvement of the proximal parts of peripheral nerves in the disease.[3] These results are in accordance with previous data that showed positive antibodies against vacuolating cytotoxin (VacA) of *H pylori* in the cerebrospinal fluid of patients with GBS, and delayed F-wave latencies in patients with GBS and positive antibodies to VacA of *H pylori* in the serum.[2] The target molecules of the specific antibody against VacA in the cerebrospinal fluid of patients with GBS are probably associated with some components of the peripheral nerve myelin, which suggests a potential role in the immune responses of patients with the demyelinating form of GBS.[4] Patients who have GBS with anti-GM1 ganglioside antibodies alone or associated with anti-*H pylori* antibodies had a significantly longer hospitalisation time to reach a low clinical score at discharge than patients who did not have anti-GM1 antibodies.



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Coloured transmission electron micrograph of the bacterium *Campylobacter jejuni*

The association between *H pylori* and autoimmunity is now well established, and GBS, an acute inflammatory polyradiculoneuropathy, is thought to be caused by autoimmune processes, triggered by preceding bacterial or viral infections, as mentioned in the Review.[1] Autoantibodies to specific neural targets have been found to impair neural function and are specific to subtypes of GBS, including acute inflammatory demyelinating polyradiculoneuropathy as shown in our study,[3] and as suggested by van Doorn and colleagues.[1] In acute inflammatory demyelinating polyradiculoneuropathy, the common epitopes of GM3, GD3, or GT3 can be shared with certain antigens localised in the peripheral nervous system that function as conduction-related molecules at the neuromuscular junction. Although studies have shown that GBS is frequently preceded by acute infections, the exact cause of GBS is not known and a specific immunological explanation has not been found. *H pylori* might induce humoral and cellular immune responses that, because of the sharing of homologous epitopes with host antigens (ie, molecular mimicry), cross-react with ganglioside surface components of peripheral nerves. Immune reactions against target epitopes in the surface membranes of Schwann cells or myelin result in GBS.[3] Molecular mimicry of host structures by the saccharide part of lipopolysaccharides of the gastrointestinal pathogens *C jejuni* and *H pylori* are thought to be associated with the development of autoimmune sequelae seen in GBS.[5] Apart from

molecular mimicry and cross-reactivity, *H pylori* infection might influence the pathophysiology of GBS through several other mechanisms, including the release of proinflammatory and vasoactive substances (eg, cytokines or eicosanoids), induction of oxidative stress, or apoptotic processes.

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We have no conflicts of interest.

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We agree with van Doorn and co-workers that more trials are needed to study the effectiveness of treatments for Guillain-Barré syndrome (GBS) on the basis of GBS outcome prognostic scores because "the course of GBS in individual patients is highly variable and the effect of treatment can be shown only by comparing groups of patients".[1] We advocate the use of more comprehensive outcome measures and earlier randomisation of patients in trials.

For an important additional statistical analysis, we advise the use of a relative score that is based on the amount of change in the absolute outcome measurement score of an individual patient during the observation period compared with the initial absolute score. This analysis would enable the effects of treatment of individual patients to be determined. The highly variable course of GBS means that some important changes seen in individual patients might be diluted by between-group comparisons, particularly for patients who have a relatively mild form of the disease. Such a relative scoring system[2] has been used for myasthenia gravis—which also has a highly variable course—in China for more than 10 years. The comparison of the proportion of the patients who meet a prespecified efficiency criteria that is based on the relative score in both treatment and placebo groups might provide us with another view on the effects of treatment, even if between-group comparisons show no group differences. GBS is a monophase disease, so we can expect that the relative score might perform better in GBS than in myasthenia gravis and even better after the "treatment-related clinical fluctuation" of GBS has been taken into account.

Our second suggestion is to stratify patients with GBS according to the delay to treatment and the outcome score on randomisation. These two factors have the most important confounding effects on the results. The interplay between these two factors might differ between trials and might vary according to different treatment mechanisms and the pathological phase of GBS. Although van Doorn and colleagues noted that outcomes were better for patients who were treated with plasma exchange early in the course of disease compared with patients treated later,[1] the opposite result was found in a comparative study of intravenous immunoglobulin and plasma exchange.[3] In this trial, the patients who were treated later tended to have a less severe form of disease and were more likely to recover spontaneously, which led to better outcome. The effects of treatment time on the results of trials of plasma exchange are considerable and might be in part caused by the simple mechanism of this treatment, which involves the removal of the pathological antibodies—the most important pathological factors in the early phase of GBS. However, the effect of timing of administration of intravenous immunoglobulin, which has many beneficial mechanisms throughout most phases of GBS, on trial results is possibly less important; this could be even less important for corticosteroids, which might have harmful effects in the early recovery phase. As well as the possible harmful inhibition of the macrophage repair processes,[1] corticosteroids might also decrease the expression of neurotrophic factors in the inflammatory cells, which might in turn impede nerve regeneration.[4] Therefore, we do not believe that

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the use of a post-hoc stratified analysis is efficient if such interactions could have occurred.

Thirdly, we believe that the course of treatment is important. In experimental allergic neuritis, an animal model of GBS, corticosteroids are beneficial when given early in the disease course.[5] However, both beneficial and harmful effects of corticosteroids can occur simultaneously in the early recovery phase; these effects could define the therapeutic window, after which there might be no benefit and further damage might even occur. As GBS is a monophasic disease, the nerve tissue in patients with GBS will repair after initial pathological insult ceases; therefore, it is important to know when the recovery phase begins to determine the treatment course. This might be accomplished by the identification of the levels of some biological markers.

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Guillain-Barré syndrome (GBS), including the Miller Fisher syndrome (MFS) subtype of GBS, and Bickerstaff's brainstem encephalitis (BBE) are immune-mediated disorders that have overlapping clinical features. Anti-GQ1b antibody is widely regarded to play a critical part in the pathogenesis of these disorders;[1] this antibody is detected in 26% of cases of GBS, 66% of cases of BBE, and in up to 95% of cases of MFS.[2] van Doorn and co-workers[3] have comprehensively reviewed the clinical features, pathogenesis, and treatment of GBS, reminding us that, despite its self-limiting nature, there is still considerable mortality and morbidity from this disorder. The findings presented by van Doorn and colleagues prompted us to re-examine the development of future therapeutic strategies for GBS.

Evidence of anti-GQ1b antibody binding to the nodes of Ranvier[4] and the presynaptic terminals of neuromuscular junctions, which leads to complement-dependent cytoxicity, has provided a great advance in our understanding of GBS, MFS, and BBE.[5] These findings have also been shown to be relevant in human beings: by use of single-fibre electromyography, patients with acute ophthalmoparesis and increased anti-GQ1b IgG antibody had abnormal jitters, which improved with clinical recovery;[6] this is the first evidence of defects in neuromuscular transmission reported in patients with MFS. Incremental responses to repetitive nerve stimulation are characteristic of defects in presynaptic neuromuscular transmission, which is seen in Lambert-Eaton myasthenic syndrome and in botulism. By use of high-frequency repetitive nerve stimulation, a defect in presynaptic neuromuscular transmission up to 3 months after clinical presentation has been shown in patients with MFS who were positive for anti-GQ1b IgG antibody.[7] This study corroborated in vitro findings of presynaptic structural defects in the nerve terminal[8] rather than a transient nerve-blocking event.

Therefore, up to this point, only anti-GQ1b antibody-associated complement activation that leads to defects in nerve terminals seems to have been validated. However, our understanding of the role of other antibodies that have been occasionally detected in these conditions[2] is still poor. It should also be emphasised that the frequency of detecting anti-GQ1b antibodies in patients with typical GBS is significantly less than in patients with MFS or BBE.[9]

The involvement of specific antibodies is unclear for acute demyelinating polyradiculoneuropathy, the most common form of GBS.[3] Until the antibodies and their targets have been identified, the consideration of upstream immunomodulation therapy is premature. Our knowledge at present suggests that development of treatment strategies could take different directions for typical GBS and MFS. Complement mediation has been recognised as the most downstream pathophysiological event so far. Therefore, treatment strategies that target the actions of complement mediation[10,11] would be a most pragmatic approach for future research.

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Authors' reply

We thank Kountouras and colleagues, Lie and Xie, and Lo for their valuable comments in response to our Review.

Kountouras and colleagues highlighted the possible role of *Helicobacter pylori* infections in Guillain-Barré syndrome (GBS). In their study, histological evidence for the presence of *H pylori* in stomach biopsy specimens was obtained by gastrointestinal endoscopy in 12 (92%) of 13 patients with acute inflammatory demyelinating polyradiculoneuropathy compared with ten (50%) of 20 control patients (p=0·02).[1] Cultures for *H pylori* were not performed in this study. On the basis of their results, an association between high levels of serum IgG anti-*H pylori* antibodies and GBS disability score at nadir was suggested.[1] Other studies have not found a relation between these serum levels and the duration of hospital admission and GBS disability score at discharge.[2] In our Review, we focused on antecedent infections associated with GBS that have been demonstrated in case–control studies and other large series of patients with GBS. Such studies are not yet available for *H pylori* and for many other types of infection for which a relation with GBS has been suggested. To further substantiate the correlation between a specific type of infection and a heterogeneous disease such as GBS, large case–control studies are required. This is even more relevant for infections that have a high prevalence in the control population, such as *H pylori*. In addition, there is an unsolved problem with the proposed mechanism by which *H pylori* could play a part in the pathology of GBS. Without specific antibiotic treatment, this infection is usually chronic and persistent, as is the case for the diseases that are associated with *H pylori*. However, GBS is a typical self-limiting disease with a subacute onset and recurrence is low: more than 95% of patients have only a single episode of GBS in their lives, even though they are not treated with antibiotics for *H pylori*. As far as we know, the researchers who have studied the role of *H pylori* in GBS have not proposed a possible mechanism to explain this observation.

We agree with Li and Xie that a relative score could be a potentially valuable method for assessing treatment effects in GBS, particularly in patients who are mildly affected by the disease. However, there are some important factors that need further discussion. First, large-scale, randomised, placebo-controlled trials to assess the effect of intravenous immunoglobulin or plasma exchange in patients who are mildly affected with GBS are needed.[3] Second, the short-term course of disease in individual patients is difficult to predict because the natural course of GBS is highly variable. Therefore, improvement after any treatment can not fully be attributed to the effect of treatment. Third, the suitability of different outcome scales needs to be understood. For example, the GBS disability scale is not useful for patients who are mildly affected by the disease because this scale is not linear and is focused on ambulation and ventilation; the Medical Research Council sumscore might be more appropriate. The evaluation of various scales that are recommended for use in patients with GBS is under investigation in the Peripheral Neuropathy Outcome Measures Standardization (PERINOMS) study.[4]

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The second suggestion by Li and Xie is to stratify patients with GBS according to the delay to start treatment and the predicted outcome at randomisation. The North American PE trial showed efficacy of plasma exchange when given to patients within the first 4 weeks from onset.[5] However, the greatest effect was seen when plasma exchange was started within the first 2 weeks.[5] A trial that compared the effect of plasma exchange, intravenous immunoglobulin, and the combination of the two included only patients who were still within the first 2 weeks since being diagnosed with GBS;[6] the results from this study suggested that patients with a slower onset of disease might be less severely affected and might have a better prognosis. Patients with a very rapid onset of disease are more likely to be treated earlier and these patients are more likely to have a worse prognosis. We agree that different treatments might have different effects during the course of disease. When developing the Erasmus GBS outcome scale, we used multivariate analysis and looked for a treatment effect of plasma exchange compared with intravenous immunoglobulin; however, this treatment effect was not statistically significantly different for the 6-month prognosis.[7]

Timing of treatment is an important factor. The optimum time point to start treatment still needs to be decided. More needs to be known about the therapeutic time window, particularly when new and potentially effective treatments, such as complement inhibitors, will be assessed in patients with GBS.

As Lo comments, the discovery of complement-dependent effects at the presynaptic membrane of mouse neuromuscular junctions by anti-GQ1b antibodies has contributed substantially to the understanding of the pathogenesis of disorders that are associated with anti-GQ1b. Electrophysiological studies in patients with these antibodies have provided additional evidence for the involvement of the neuromuscular junction; therefore, anti-GQ1b antibody and complement-mediated destruction of the neuromuscular junction or other sites of the peripheral nerve are likely to be relevant in patients with Miller Fisher syndrome and in other patients with GBS who are positive for anti-GQ1b. However, as the author indicated, the frequency of anti-GQ1b antibodies in patients with GBS who do not have ophthalmoplegia or bulbar weakness is generally low.

There is, however, experimental evidence that antibodies to other gangliosides also have complement-dependent neurotoxic effects. Antibodies to GD1a induce similar complement-dependent damage to motor nerve terminals in mice overexpressing GD1a.[8] Antibodies to GM1 in rabbits lead to complement activation at the nodes of Ranvier and concomitant disruption of the nodal structure and loss of local voltage-gated sodium channels.[9] Whether these effects also occur in patients is not yet known. Single-fibre studies have found evidence for dysfunction at the motor-nerve endplates and axonal membrane in patients with GBS who have antibodies to GM1, GM2, GD1a, and GD1b, and even in patients without these anti-ganglioside antibodies.[10] The extent to which these abnormalities at the neuromuscular junction might contribute to the neurological deficit is not yet known.

The question of whether antibody-mediated and complement-mediated effects are a general neurotoxic mechanism in GBS is highly relevant for the choice of future therapies. The target of the immune response in most patients with acute demyelinating polyradiculoneuropathy is still unknown. Patients might have antibodies to ganglioside complexes or other peripheral nerve structures that have not yet been investigated; however, other components of the immune system could also play a part in the pathogenesis of these disorders. Autopsy studies in cases of acute demyelinating polyradiculoneuropathy have shown depositions of membrane attack complex at the myelin membranes.[11] Further evidence for the role of complement in the pathogenesis of GBS will be difficult to obtain, except by therapeutic studies of complement inhibitors in patients.[12] Given the evidence for complement activation in patients with Miller Fisher syndrome and acute motor axonal neuropathy, and the circumstantial support for complement activation in other variants of GBS, this is certainly an appealing strategy.

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PAvD has received a consultation fee for being in the steering committee of the ICE trial (sponsored by Talecris). PAvD's department has received a research grant from Baxter Healthcare. Both Talecris and Baxter are manufacturers of IVIg. BCJ and LR have no conflicts of interest.

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Erratum

Don Gilden. Treatment of Bell's plasy—the pendulum has swung back to steroids alone. The Lancet Neurol *2008; 7: 977.* Owing to an editorial error, the picture that accompanied this comment was incorrect and did not show a patient with the signs of Bell's palsy. We apologise for this inaccuracy.