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Clinical Evaluation of a Ten-Day Regimen with Esomeprazole, Metronidazole, Amoxicillin, and Clarithromycin for the Eradication of *Helicobacter pylori* in a High Clarithromycin Resistance Area

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Keywords
H. pylori treatment, first line, second line, concomitant therapy, clarithromycin resistance, dual resistance.

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Abstract

Background: Increasing clarithromycin resistance reduces *Helicobacter pylori* eradication rates with conventional triple regimens. We evaluated effectiveness and safety of a 10-day-quadruple nonbismuth containing regimen, as first-line treatment or second-line treatment (after conventional triple) for *H. pylori*, and assessed impact of antibiotic resistance on treatment success.

Materials and methods: Eligible patients had upper GI endoscopy and positive CLO-test, also confirmed by histology and/or culture. The eradication scheme comprised: Esomeprazole 40 mg, Metronidazole 500 mg, Amoxicillin 1000 mg, and Clarithromycin 500 mg, twice daily, for 10 days. Treatment adherence and adverse effects were recorded. Eradication was tested by ^{13}C-urea breath test or histology.

Results: One hundred and ninety out of 198 patients (115M/83F, aged 18–81, mean 52 years, 37% smokers, 27% ulcer disease) who completed the study protocol were evaluated for eradication. Adherence to treatment was 97.7% (95% CI 95.9–99.6). Six (3.2%) patients experienced severe side effects and discontinued treatment. Intention to treat and per protocol analysis in first line was 91.5% (95% CI 86.2–94.8) and 95% (95% CI 90.4–97.4) and in second line was 60.6% (95% CI 43.6–75.3) and 64.5% (95% CI 46.9–78.8), respectively. Antibiotic susceptibility tests were performed in 106 of 124 (85%) patients who gave consent. Among them 42 (40%) harbored clarithromycin resistant strains. Eradication rates were significantly higher in sensitive and single clarithromycin or metronidazole resistant (37/37, 100% and 43/47, 91%) than in dual resistant strains (12/22, 55%) ($p < .0001$). Specifically, concomitant regimen eradicated 7/10, 70% of dual resistant strains as first-line treatment and 5/12, 42% as second-line treatment. Multivariate analysis showed that dual resistance was the only independent significant predictor of treatment failure.

Conclusions: The 10-days "concomitant" regimen is effective and safe first-line *H. pylori* treatment, in a high clarithromycin resistance area, although dual antibiotic resistance may compromise its effectiveness.

Helicobacter pylori (*H. pylori*), a global human pathogen which affects approximately 50% of the world's adult population, is playing a cardinal role in the development of peptic ulcer disease and gastric malignancy [1]. Recommended triple therapies that yielded high efficacy more than a decade ago [2] are currently been associated with unacceptably low eradication rates (i.e., <80%) in most European countries and the USA [3,4]. This is mainly due to increasing prevalence of *H. pylori* resistance to clarithromycin (CLA), the key antibiotic in triple therapies [5,6].

Current European guidelines preclude the use of CLA-based triple therapies as first-line eradication treatments in areas with high prevalence of CLA resistance

(i.e., >20%) without a prior antibiotic susceptibility test [7]. Instead, they recommend a bismuth containing quadruple regimen (i.e., a proton-pump inhibitor (PPI) with bismuth salts, tetracycline (TET), and metronidazole (MET)) as first-line treatment although it doesn't seem to exceed standard triple therapy eradication rates [8–10]. Alternative first-line treatments, in high CLA resistance areas according to the recent Maastricht-4 guidelines, are two nonbismuth containing quadruple regimens, the so-called sequential and concomitant, which include a PPI with Amoxicillin (AMO), MET, and CLA, given twice daily, for 10 days, in a sequential or concomitant manner, respectively [7]. Evaluation of these therapies in a low CLA resistance area proved very efficacious [11], but this was questioned in areas presenting higher prevalence of CLA and/or MET resistance [12–15]. In a recent two-center open-label study in Greece, we reported excellent overall *H. pylori* cure rates with the use of ten-day concomitant as first-line treatment (94.5% on per protocol and 91.6% on intention to treat analysis) [16]. Moreover, in another study including patients from a center where standard triple therapy was performing fairly well few years before, concomitant was significantly more efficient than standard triple therapy of the same duration [17,18].

In the present study, we aimed to evaluate the effectiveness, safety, and tolerability of a ten-day concomitant regimen, as first- or second-line treatment, in a population with relatively high resistance to CLA and MET and assess the impact of antibiotic resistances on eradication rates.

Methods

Patients

This is a prospective, open-label single-center study, undertaken in a clinical setting. From January 2010 to June 2012, we evaluated endoscopically 510 consecutive patients presenting with upper GI symptoms and/or iron deficiency anemia (Fig. 1). Among them 230 (45%) were infected with *H. pylori*. Infection was established by a positive rapid urease test (CLO-test) and later confirmed by histology and/or culture. One hundred and ninety-eight patients, who met the inclusion criteria and gave written informed consent, were assigned to receive a four-drug concomitant regimen comprising esomeprazole 40 mg before meals, MET 500 mg, AMO 1 gr, and CLA 500 mg, after meals, all taken twice daily, for a total of 10 days. All medications were prescribed to patients who obtained them in the pharmacy. One hundred and twenty-four of 198 (63%) patients also consented to send two antral biopsies for

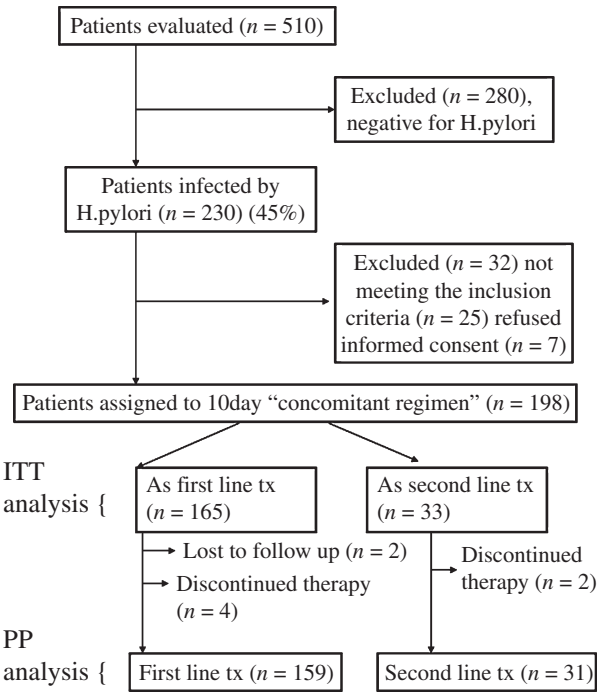

Figure 1 Flow diagram of the progress of patients through the study.

culture and antibiotic susceptibility tests. All patients had an endoscopy-based diagnosis of peptic ulcer disease, nonulcer dyspepsia with or without mild gastroesophageal reflux disease (i.e., nonerosive reflux disease, esophagitis of first or second degree according to Los Angeles classification system). Exclusion criteria were: age under 18 years, presence of severe comorbidities (i.e., liver cirrhosis, renal failure, severe cardiovascular or pulmonary diseases), previous gastric surgery, gastric or other malignancies, known allergy to the study medications, severe gastroesophageal reflux disease (i.e., severe esophagitis, Barrett's esophagus, esophageal strictures or ulcers), use of antibiotics, bismuth salts, NSAIDs or aspirin (ASA) in the preceding month and use of PPIs in the preceding 2 weeks, pregnancy or lactation, and not willing to participate in the study. Previous *H. pylori* eradication treatment was not an exclusion criterion providing that the patient was still infected. In this latter group (33 patients), the regimen under evaluation was prescribed as a second-line therapy. A careful medical history and a clinical examination were performed prior to inclusion. The study protocol was approved by the scientific board of our institution and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Side effects of treatment were assessed on a structured clinical interview with a specific questionnaire completed immediately after the end of concomitant

therapy and at the final re-evaluation. During the interview, patients were asked to grade the severity of every adverse event experienced as "mild" (transient and well tolerated), "moderate" (causing discomfort and partially interfering with common everyday activities), or "severe" (causing considerable interference with patients daily activities). Incapacitating or life threatening complications were classified as serious and required reporting to regulatory agency (National Organization of Medicines). Adherence to treatment was assessed by providing all patients with a prestructured printed table with all dosages illustrated, asking to tick each time when a pill was consumed and bring it back along with any tablet not consumed, for pill counting. In case of discrepancies found between the structured printed table and residual medication, the latter was taken into account to measure patient's adherence. Poor adherence was considered when <90% of the total medication was taken. Efficacy of treatment was evaluated by ^{13}C-urea breath test (^{13}C-UBT) performed according to the standard European protocol at 4–6 weeks after the end of antibiotic treatment [19]. In patients requiring a follow-up endoscopy due to gastric ulcer (3.6%), the diagnostic test of choice was histologic examination of four samples taken from the antrum and body and stained by modified Giemsa. In the post-treatment period, symptomatic patients were allowed to use only antacids on demand except all cases with esophagitis or gastric ulcer disease where patients were allowed to continue esomeprazole 40 mg once daily for another 3 weeks and stopping it at least 2 weeks before re-evaluation.

Sample Size Calculation

Concomitant treatment given as first line was estimated to increase *H. pylori* eradication rate, from about 73% reported recently using standard triple therapy in high CLA resistance areas [17] to the internationally accepted rate of 90% [3]. To achieve a power of 80% with a type I error of 0.05, the number of patients with culture and antibiotic susceptibility test needed was calculated to be 80. However, to achieve this number of patients with positive cultures, we accepted a 15% missing rate due to culture failure (negative cultures) and increased the number to 92.

Culture and Antibiotic Susceptibility Tests

Isolation of clinical strains

All biopsies were aseptically placed in thioglycollate medium (Oxoid, Basingstoke, UK) and were processed for *H. pylori* isolation within 2–4 hours after endoscopy. Biopsy samples were vigorously vortexed with addition of sterile glass beads and cultured for up to 7 days, at 37 °C under microaerophilic conditions (CampyPak-Plus, Becton-Dickinson, Cockeysville, MD.), on Columbia agar plates containing antibiotics (vancomycin 10 μg/mL, trimethoprim 10 μg/mL, polymyxin B 10^4 IU/L, amphotericin B 2 μg/mL, nalidixic acid 10 μg/mL, bacitracin 30 μg/mL, and fluorocytosine 5 μg/mL), supplemented with 7% v/v horse blood and 1% v/v Vitox (Oxoid, Basingstoke, UK). Culture sweeps, as well as individual colonies, were collected and frozen at −80 °C, until used.

Antibiotic susceptibility testing

Antibiotic susceptibility was tested on Mueller Hinton agar medium (Beckton Dickinson) supplemented with 10% horse blood utilizing E-test strips (AB bioMirieux, Solna, Sweden). Agar plates were freshly prepared and used within 7 days following their preparation. Bacterial inoculum was prepared from a 1 or 2-day-old culture, and bacterial density was adjusted to McFarland 3. Motility and morphology of the bacteria were always examined prior to inoculation, by phase contrast microscopy, to avoid the presence of coccoid forms. E-test strips were applied with sterile forceps to the dried agar surface, following application of the bacterial inoculum. Plates were incubated at 37 °C under microaerophilic conditions. Results were read after 72 hour of incubation. For interpretation of the results, MIC (μg/mL) cut-off values used were Clarithromycin: >1, Levofloxacin: >0.5, Tetracycline: >1, Metronidazole: >8, and Amoxicillin: >0.5, according to the standard European protocol [20]. Throughout the study two reference strains, namely CCUG 38771-susceptible to amoxicillin, clarithromycin and metronidazole and CCUG 38772 -susceptible to amoxicillin, but resistant to clarithromycin (1 μg/mL) and metronidazole (32 μg/mL), were included for quality control of antibiotic resistance breakpoints.

Statistical Analysis

We used the statistical program SPSS version 13 (SPSS, Chicago, IL) to evaluate results. Comparisons of proportions were done using the chi-square or Fisher's exact test. Continuous nonparametric data were compared using the Mann–Whitney *U*-test. All continuous variables are expressed as the mean ± 1SD and categorical variables as absolute numbers and relative frequencies. Stepwise multivariate logistic regression analysis was performed to evaluate factors influencing *H. pylori*

eradication in patients under first-line concomitant treatment who had positive culture and an antibiotic susceptibility test. As independent variables, we used the presence of single or dual resistances to CLA and/or MET, age over or under 50 years, gender, alcohol use of more than 21units/week for males and 14 for females, smoking, the presence of ulcer or nonulcer disease. All *p*-values of <.05 were considered significant.

Results

Of a total of 510 consecutive patients referred to our department for gastroscopy, during the study period, 230 (45%) were diagnosed with *H. pylori* infection. Among them two were excluded because of gastric and esophageal adenocarcinomas, one was excluded because of gastric MALT lymphoma, six were excluded because of severe comorbid diseases (one liver cirrhosis, one chronic renal failure under hemodialysis, one brain cancer, and three with severe cardiovascular and/or pulmonary diseases). Another two patients were alcohol and/or drug addicted, one patient reported recent use of antibiotics, eight chronic use of NSAIDs, aspirin and/or recent use of PPIs, one patient reported a confirmed allergy to penicillin, one had a previous subtotal gastrectomy, and three patients had severe esophagitis or Barrett's esophagus. Finally, seven patients denied a signed consent. The clinical and demographic data of the remaining 198 patients included are presented in Table 1. Of them, 165 were naive to previous eradication treatment and 33 had a previous *H. pylori* eradication failure (31 had received PPI/CLA/AMO, and 2 PPI/CLA/MET for 10 days). Among the patients received concomitant as first-line treatment, two were lost to follow-up and another four discontinued treatment prematurely because of severe adverse events. These patients were included in the intention to treat analysis and considered as treatment failures. The remaining 159 patients who completed the treatment protocol and returned for re-evaluation were included in the per protocol analysis. Similarly, among the 33 patients who received the concomitant as a second-line therapy, two discontinued treatment prematurely because of severe side effects and 31 completed the protocol and returned for re-evaluation.

Table 1 Characteristics of patients studied

Treatment: 10-day Concomitant	First line (n = 165)	Second line (n = 33)
Demographic data		
Gender M/F n (%)	98/67 (59.3/40.6)	17/16 (53.3/46.7)
Age (yrs) mean (1SD)	52 (14.3)	51.5 (12.4)
Smokers n (%)	61 (36.9)	7 (21)
Alcohol consumption >21/w, males >14/w, females	10 (6)	1 (3)
Endoscopic findings		
Non ulcer dyspepsia, n (%)	121 (73.3)	33 (100)
Gastric ulcer, n (%)	6 (3.6)	0
Duodenal ulcer, n (%)	38 (23)	0

H. pylori Eradication Rates and Adverse Events

Efficacy of concomitant therapy was 91.5% (95% CI 86.2–94.8) and 95% (95% CI 90.4–97.4) as first line and 60.6% (95% CI 43.6–75.3) and 64.5% (95% CI 46.9–78.8) as second line in intention to treat and per protocol analysis, respectively. Analytical results of *H. pylori* eradication with concomitant therapy are shown in Table 2. Eight patients who failed *H. pylori* eradication with first-line concomitant therapy were effectively treated (100% eradication rate) with a 10-day second-line regimen based on Levofloxacin (LEV) (PPI with LEV 500 mg and AMO 1gr, bid).

Adverse events were recorded in 80 of 198 (40%) patients but were mostly mild or moderate in severity. In general, patients in the second-line concomitant therapy group experienced more frequently side effects (55% vs 38%). The frequency and severity of each one of the reported side effects are shown in Table 3. Taste disturbances and diarrhea were, by far, the most frequent complaints reported by 26.3% and 13.6% of cases, respectively. Less frequent were dizziness (5%) and other gastrointestinal disturbances like bloating (6%), epigastric pain (4.5%), nausea (3.5%), vomiting (3%), and stomatitis or glossitis (3%). Adverse effects like cardiac arrhythmia and allergic rash were only infrequently recorded (0.5% each). Overall 6 patients

Table 2 *H. pylori* eradication rates of the 10 day concomitant treatment presented in first- and second-line treatment groups

H. pylori eradication	ITT (%)	PP (%)	*p*-value
First line	151/165 (91.5)	151/159 (95)	
Without culture	76/84 (90.5)[a]	76/79 (96.2)[b]	.8[a]
With culture	75/81 (92.6)[a]	75/80 (93.7)[b]	.7[b]
Second line	20/33 (60.6)	20/31 (64.5)	
Without culture	5/5 (100)[a]	5/5 (100)[b]	.1[a]
With culture	15/28 (53.6)[a]	15/26 (57.7)[b]	.1[b]

ITT, intention to treat analysis; PP, per protocol analysis.
[a]Comparisons between groups with and without culture in ITT analysis.
[b]Comparisons between groups with and without culture in PP analysis.

Table 3 Adverse effects presented during treatment with the 10-day concomitant eradication regimen

Adverse effect – severity	First line 62 (37.6%)	Second line 18 (54.5%)
Anorexia	2 (1.2%) – 2/2	2 (6.1%) – 2/1
Nausea	5 (3%) – 2/1, 3/2	2 (6.1%) – 2/2
Vomiting	5 (3%) – 2/2, 3/3	1 (3%) – 1/3
Taste disturbances	36 (21.8%) – 25/1, 10/2, 1/3	16 (48.5%) – 16/1
Dizziness	8 (4.9%) – 2/1, 5/2, 1/3	2 (6.1%) – 1/2, 1/3
Vertigo	1 (0.6%) – 1/2	0
Epigastric pain	8 (4.9%) – 2/1, 4/2, 1/3	1 (3%) – 1/3
Diarrhea	25 (15.2%) – 18/1, 6/2, 1/3	2 (6.1%) – 2/1
Bloating	10 (6.1%) – 5/1, 4/2, 1/3	2 (6.1%) – 2/1
Headache	3 (1.8%) – 2/1, 1/2	2 (6.1%) – 2/2
Rash	1 (0.6%) – 1/1	0
Itching	1 (0.6%) – 1/1	2 (6.1%) – 2/2
Cardiac arrythmia	1 (0.6%) – 1/1	0
Colpitis or posthitis	4 (2.7%) – 3/2, 1/3	0
Stomatitis or glossitis	6 (3.6%) – 2/1, 4/2	0
Total	116	32

Adverse effects are expressed in absolute number and relative frequency (%) and severity in absolute number and grade (1 = mild, 2 = moderate, 3 = severe).

(3%) (four in the first-line group and two in the second-line group) discontinued treatment prematurely because of severe side effects namely, severe bloating, epigastric pain and vomiting, dizziness, and troublesome valanoposthitis with coexisting mild cardiac arrhythmia (transient supraventricular extrasystoles). No adverse effect was fatal or required hospitalization. All events experienced by the patients disappeared as soon as the eradication treatment was stopped or discontinued. Adherence to treatment was excellent and comparable among first- and second-line treatment groups (98.5%, 95% CI 97–99% vs 96.3%, 95% CI 90–100%).

Antibiotic Resistance Patterns and Impact on *H. pylori* Eradication Rates

Helicobacter pylori resistance patterns in patients without and with a previous *H. pylori* eradication trial are illustrated in Table 4. Primary resistance to CLA and MET was recorded in 25 (31%) and in 35 (43.7%) of 80 patients, respectively. Among them 10 patients (12.5%) harbored resistant strains to both CLA and MET (dual resistant). Primary resistance to LEV was recorded in only eight patients (10%), five of whom were also resistant to either CLA (1) or MET (3) or both (1). In contrast, remarkably higher percentages of CLA, MET, and LEV resistance (65%, 54%, and 27%, respectively) were recorded in the group of patients taking concomitant as second-line therapy. Finally, no resistance to AMO or TET was recorded in our study population.

Table 4 Prevalence of antimicrobial resistance in first- and second-line treatment groups

Antibiotic	First line (n = 80)	Second line (n = 26)
MET (%)	35/80 (43.7)	14/26 (54)
CLA (%)	25/80 (31)	17/26 (65)
MET + CLA (%)	10/80 (12.5)	12/26 (46)
LEV (%)	8/80 (10)	7/26 (27)
AMO	0/80	0/26
TETR	0/80	0/26

MET, metronidazole; CLA, clarithromycin; LEV, levofloxacin; AMO, amoxicillin; TETR, tetracycline.

Overall eradication rates were significantly higher in sensitive and single clarithromycin or metronidazole resistant strains (37/37, 100% and 43/47, 91%) than in dual resistant strains (12/22, 55%) ($p < .0001$). Concomitant regimen given as first-line therapy eradicated all 30 CLA and MET sensitive (dual sensitive), all 25 single resistant to MET and all eight LEV resistant *H. pylori* strains. Compared to dual sensitive, this regimen eradicated significantly less *H. pylori* strains with single CLA (13 of 15, 87%, $p < .015$) and dual (CLA and MET) (7 of 10, 70%, $p < .02$) resistance. The effect of single CLA, single MET, and dual resistance on *H. pylori* eradication rates with concomitant therapy and the relative comparisons are shown in Table 5. In

Table 5 Eradication rates in first- and second-line treatment with concomitant regimen according to *H. pylori* antibiotic susceptibility pattern

Susceptibility pattern	First line (n = 80) (%)	*p*-value	Second line (n = 26) (%)	*p*-value
MET^S CLA^S	30/30 (100)		7/7 (100)	
MET^R CLA^S	25/25 (100)	1[a]	1/2 (50)	.4[b]
MET^S CLA^R	13/15 (87)	.015[a]	4/5 (80)	.8[b]
MET^R CLA^R ($dual^R$)	7/10 (70)	.02[a]	5/12 (42)	.04[b]
LEV^S	67/72 (93)		13/19 (68)	
LEV^R	8/8 (100)		4/7 (57)	

MET, metronidazole; CLA, clarithromycin; LEV, levofloxacin; S, sensitive; R, resistant.

[a]Comparison was done with first-line MET^S CLA^S group.

[b]Comparison was done with second-line MET^S CLA^S group.

contrast, only 10 of 19 (52%) *H. pylori* strains exhibiting single or dual resistance to MET and/or CLA were eradicated by second-line concomitant therapy, whereas much better results were achieved in sensitive *H. pylori* strains (7 of 7, 100%). Analytical results for first- or second-line concomitant therapy in all cultured *H. pylori* strains according to their susceptibility patterns are illustrated in Table 5.

Univariate and Multivariate Analyses for Evaluation of Factors Influencing *H. pylori* Eradication in First-Line Treatment

All univariate analyses performed to evaluate factors that could possibly influence *H. pylori* eradication in patients under first-line concomitant treatment who had positive culture and an antibiotic susceptibility test (n = 80) showed to be nonsignificant except from the presence of dual resistance to CLA and MET as shown in Table 6.

Similarly in the stepwise multivariate logistic regression analysis using treatment success as the dependent variable and as independent variables single or dual resistance to CLA and/or MET, age over or under 50 years, gender, alcohol use of more than 21 units/week for males and 14 for females, smoking, presence of ulcer or nonulcer disease, the only independent factor that influenced significantly outcome of concomitant therapy was the presence of dual resistance (odds ratio = 0.0686, 95% CI 0.009–0.48, p = .007).

Table 6 Univariate analysis of all variables evaluated with respect to eradication outcome in 80 patients who had concomitant treatment as first line, positive cultures and antibiotic susceptibility tests

	Correlations	
Variable	r	*p*-value
Age (over or under 50 years)	0.11	.3
Gender	0.12	.2
Alcohol use (Males >21, Females >14 Units/Week)	0.04	.7
Smoking	0.05	.6
Ulcer or nonulcer disease	0.08	.4
Single CLA resistance	0.14	.2
Single MET resistance	0.17	.1
Dual (MET and CLA) resistance	0.37	.0007[a]

MET, metronidazole; CLA, clarithromycin.
[a]significant.

Discussion

The continuous rise of bacterial resistance to CLA gradually reduces *H. pylori* eradication rates with standard triple regimens. In Greece, where bacterial resistance to CLA and MET is relatively high (above 20% to CLA and close to 40% to MET) [20,21], the conventional triple therapy has already shown low eradication rates and should be abandoned as first-line treatment [17]. In this study, we used a nonbismuth quadruple regimen, the so-called "concomitant", by simply adding MET 500 mg bid on the conventional triple therapy of the same duration (i.e., 10 days). The latter regimen has recently been proposed by the last (Maastricht–4) European consensus as alternative to bismuth quadruple first-line treatment in areas where the level of CLA resistance exceeds 20% [7]. We choose concomitant therapy over bismuth quadruple because *H. pylori* eradication rates with the latter were unacceptably low when used as first-line treatment in Greece while bismuth therapy containing monocapsule is not available [22,23]. Contradictory results were also reported even when this regimen was used as second-line therapy [24,25]. On the other hand, the nonbismuth sequential therapy which is another alternative to bismuth quadruple regimen has been reported equally effective to concomitant but only in areas with low levels of CLA or dual resistance [11], whereas in high (CLA and/or MET) resistance areas its effectiveness remains questionable [12,15].

We evaluated concomitant therapy in a clinical practice setting, mainly as first-line treatment but also as second-line treatment, in a small group of patients who had failed to eradicate *H. pylori* with conventional triple therapies. Our results clearly indicate that concomitant gives high eradication rates as first-line treatment but not as second-line treatment. To our knowledge, this is the first study that evaluated the effectiveness of 10-day concomitant as second-line therapy in consecutive patients but the results were disappointing, probably because of the extremely high rates of antibiotic resistances observed in these difficult-to-treat patients. Much better eradication rates (88, 5% ITT, and 94% PP) were obtained with a 7-day second-line concomitant regimen including rabeprazole, in a randomized study from Japan, where very low rates of MET resistance (5%) were recorded [26]. On the other hand, first-line treatment with the "concomitant" regimen performed quite well producing eradication rates over 90% in ITT and close to 95% in PP analysis. Similar results have been recently reported with 10-day concomitant therapy by another study from Spain which included patients with relatively high bacterial resistance rates to CLA (20%), MET (33%), or both (10%) [27]. In the latter study, peptic ulcer disease

proved a significant predictor of treatment success. According to our results, neither ulcer disease nor any other factor studied except dual resistance has proved to influence *H. pylori* eradication rates. This discrepancy might be caused by different percentages of Cag-A positive *H. pylori* strains in nonulcer dyspepsia patients among the two populations, as these are the strains reported to be less amenable to eradication with conventional *H. pylori* therapies [28,29].

The high overall efficacy of our study regimen is probably due to high eradication rates produced on single resistant bacterial strains. This regimen eradicated 96% of the single resistant strains. Comparable eradication rates have recently been recorded in a meta-analysis using sequential therapy in MET (96%) and CLA (75%) resistant strains [13]. However, other studies have shown significantly decreased eradiation rates with sequential therapy for CLA resistant *H. pylori* strains, using either standard culture and E-test (57%) or the detection of point mutations in 23s RNA gene by PCR (65% resistant vs 98% sensitive) [30,31]. Finally, a large multicenter study from Taiwan found that both single CLA and MET resistance to be independent prognostic factors of sequential therapy failure [32]. Similarly, in our study, the eradication rate for single CLA resistant strains was lower (87%) than for dual sensitive and single MET resistant strains (100%) but not as low as reported for sequential therapy. However, this result has not influenced overall treatment success as was proved in our multivariate analysis. Our results have clearly shown that this regimen could be assigned as first-line treatment in high resistance areas because it overcomes both single CLA and MET resistances. In addition, the high overall efficacy of concomitant therapy on sensitive (100%) or single resistant strains (96%) protects from a gross dissemination of secondary *H. pylori* resistances in the community.

The only factor adversely affected the outcome of concomitant therapy, in this group of patients, was the presence of dual bacterial resistance to MET and CLA. According to the results on dual resistant strains from two previous comparative studies, concomitant seemed to work better than sequential therapy [11,27]. However, the number of strains was limited and a type 2 error is possible. Certainly, we need much more data on the efficacy of both therapies on dual resistant strains, preferably in the context of randomized controlled studies, before firm conclusions could be drawn. In the present study, we have been able to culture *H. pylori* and extract susceptibility data on an adequate number of naïve patients (80) treated with 10-day concomitant therapy. Dual bacterial resistance to MET and CLA was found in 10 (12.5%) patients**.** This influenced concomitant therapy, although not drastically, eradicating the majority (70%) of such strains as first-line therapy. This percentage is much greater than the expected by single effect of the remaining drugs (PPI and AMO 1gr bid for 10 days) [33]. Thus, it could be speculated a synergistic effect among all three antibiotics along with the additive effects of double dose of a powerful new generation PPI (esomeprazole) included in this regimen [34,35]. In extremely high rates of dual resistance as was the case (46%) in patients who took concomitant as second-line therapy, this treatment could no longer be efficient at least without a prior antibiotic susceptibility test.

Finally, as expected, LEV resistance had no impact on eradication rates with concomitant therapy as opposed to LEV-based first-line treatments [12,36,37]. This drug should be better reserved as substitute for CLA in triple second-line therapy at least in areas like ours with low resistance (<10%) to LEV [21,38,39].

Adherence to treatment was excellent with concomitant therapy, in accordance with recently published series [27]. Only a few patients experienced severe side effects leading to premature discontinuation of treatment (between the 5th and 7th day) but all who had concomitant as first-line therapy, finally eradicated the bacterium. It is questionable whether concomitant given for 5 days could be as effective as the 10-day regimen. A recent pilot study conducted in Thailand suggested that the 5-day regimen produced eradication rates below the accepted level of 90% [40]. Also, in another multicenter study from Latin America very low eradication rates were recorded with 5-day concomitant therapy [15].

Limitations of our study were the absence of culture and antibiotic susceptibility tests in a considerable number of our patients although demographics and *H. pylori* cure rates were comparable among patients who consented for culture and those who did not, either on first- or second-line treatment. In addition, our group may not be representative of the whole Greek population in terms of antibiotic susceptibility patterns. Discrepancies of metronidazole resistance between E-test and the agar dilution method have been reported previously [41]. In our study, however, high MIC values have been determined for metronidazole resistance (>64 μg/mL) in most cases, minimizing the possibility of falsely reporting eradication rates for MET resistant strains of *H. pylori*. Nevertheless, we were able to subsequently confirm our E-test-based susceptibility data using agar dilution (as a reference), although MET resistance tends to be overestimated by E-test compared to agar dilution, especially at higher resistance levels.

In conclusion, 10-day nonbismuth quadruple "concomitant" regimen represents effective and safe first-line treatment option for *H. pylori*, even in populations with relatively high bacterial resistances to MET and/or CLA. The only independent prognostic factor for treatment failure with concomitant therapy was the presence of dual bacterial resistance to both CLA and MET, although the latter factor seems not to be detrimental for this regimen at the primary dual resistance level recorded in our study.

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Competing interest: the authors have no competing interests.

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Presence of Terminal EPIYA Phosphorylation Motifs in *Helicobacter pylori* CagA Contributes to IL-8 Secretion, Irrespective of the Number of Repeats

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Abstract

CagA protein contributes to pro-inflammatory responses during *H. pylori* infection, following its intracellular delivery to gastric epithelial cells. Here, we report for the first time in an isogenic background, on the subtle role of CagA phosphorylation on terminal EPIYA-C motifs in the transcriptional activation and expression of IL-8. We utilized isogenic *H. pylori* mutants of P12 reference strain, expressing CagA with varying number of EPIYA-C motifs and the corresponding phosphorylation defective EPIFA-C motifs while preserving intact the CM multimerization motifs. These mutants had been previously closely scrutinized in terms of type IV secretion system functionality, CagA translocation and its subsequent phosphorylation. Following infection of gastric epithelial cell lines, transcriptional activation of IL-8 gene and secreted IL-8 levels were found to be strictly dependent upon the functionality of the EPIYA-C phosphorylation motifs, as EPIFA-C phosphorylation-deficient CagA expression failed to induce full IL-8 transcriptional activity. Interestingly, levels of IL-8 gene activation and of secreted IL-8 were the same, irrespective of the number of EPIYA-C terminal repeats. We monitored IkBα phosphorylation and confirmed CagA involvement in NF-kB activation. Furthermore, we observed that presence of EPIYA-C functional phosphorylation motifs contributed to NF-kB activation. NF-kB upstream signaling events, such as early ERK1/2 and AKT activation were confirmed to be independent of EPIYA-C phosphorylation. On the contrary, use of TAK1 specific inhibitor 5Z-7-Oxozeaenol resulted in complete arrest of IL-8 secretion, in a dose-dependent manner, irrespective of CagA status. *H. pylori*-infected TAK1$^{-/-}$ mouse embryonic fibroblasts (MEFs) failed to induce NF-kB activity, unlike the respective control MEFs. CagA and TAK1 were found to immunoprecipitate together, irrespective of CagA EPIYA-C status, thus confirming earlier reports of TAK1 and CagA protein interaction. Our data suggest that CagA may potentially interfere with TAK1 activity during NF-kB activation for IL-8 induction in early *H. pylori* infection.

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Introduction

Helicobacter pylori (*H. pylori*) colonize the gastric mucosa of 35–70% of people worldwide and remain the main etiologic factor for development of chronic active gastritis and peptic ulcer [1,2]. However, infection is usually asymptomatic in the vast majority of hosts, as virtually all carriers develop superficial chronic active gastritis, whereas only about 10% will suffer gastric or duodenal ulceration and 0.5–2% will develop gastric adenocarcinoma or B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) [3]. The presence of neutrophil polymorphs in a background of chronic inflammation remains the hallmark of *H. pylori* infection, because neutrophil activity is an almost universal phenomenon in *H. pylori* gastritis [4]. Neutrophils can be found in biopsy specimens, in virtually all cases of *H. pylori*-positive patients and they are a very sensitive indicator of the presence or absence of *H. pylori* because they disappear within days of cure of infection [4,5]. Interleukin 8 (IL-8), a CXC chemokine specific for neutrophil granulocyte chemotaxis, has been shown to be upregulated in *H. pylori* infected patients [5,6] and to correlate with histological severity of gastritis [7]. Early reports have linked elevated inflammatory response and cytokine expression *in vivo*, to *cagA*-positive *H. pylori* strains [8]. CagA protein is a major *H. pylori* virulence factor which has gained much interest from a clinical point of view, as a marker of *H. pylori*-associated disease, having been shown to confer increased risk for development of atrophic gastritis, peptic ulcer, gastric cancer and lymphoma [1,3,9,10]. From a mechanistic standpoint, CagA activity in gastric epithelial cells has been shown to be associated with disruption of intercellular junctions and of gastric epithelial cell polarity, increased cell motility and elongation cellular phenotypes, as well as propagation of signaling pathways relating to proliferation and inflammation in the gastric epithelium [11]. To this effect, CagA has been reported to interact with a number of intracellular key molecules, such as Shp2, Ras GTPase activating protein (Ras-

GAP), phosphoinositide-3 (PI3)-kinase, and the adaptor proteins Crk, Grb2 and Grb7 [12–15].

The gene coding for CagA resides at the end of the cag Pathogenicity Island (cagPAI), a gene cluster of 40 Kbps that altogether codes for a type IV secretion system (T4SS), via which CagA, is translocated from the epithelium-adhered bacteria into gastric epithelial cells [16]. Early reports clearly correlate IL-8 induction in gastric epithelial cells to a functional cagPAI [17]. Once inside the cell, CagA is phosphorylated by host cellular kinases Src [18,19] and Abl [20,21], on repeating Glutamic Acid-Proline-Isoleucine-Tyrosine-Alanine (EPIYA) tyrosine phosphorylation motifs located at the carboxyl terminus of the protein. EPIYA motifs found in *H. pylori* strains of Western origin constitute three distinct types according to the surrounding aminoacid sequence, namely EPIYA-A (*EPIYA*KVNKKK(A/T/V/S)GQ), EPIYA-B (*EPIY(A/T)*(Q/K)VAKKVNAKI) and terminal EPIYA-C (*EPIYA*TIDDLG) [22]. These motifs vary considerably among strains regarding both the type and number, which may account for the differences observed in the pathogenic potential of *H. pylori* [23]. Hierarchic intracellular phosphorylation of CagA has been recently demonstrated, with c-Src only phosphorylating EPIYA-C motifs, whereas c-Abl phosphorylating EPIYA-A, EPIYA-B, and EPIYA-C [24]. Moreover, further analysis revealed that CagA molecules were phosphorylated on 1 or 2 EPIYA motifs, but never simultaneously on 3 motifs [24]. The number of EPIYA-C motifs has been suggested to determine the levels of CagA phosphorylation [25], to induce increasing levels *H. pylori*-related cytoskeletal rearrangements in epithelial cells *in vitro* [25,26], and to confer oncogenic potential [22]. However, we have reported that in children [27] and in adult patients [28] the number of CagA EPIYA-C motifs does not correlate to more severe inflammatory response in the lamina propria. Moreover, in adults the presence of one CagA EPIYA-C site was found to be an independent risk factor for presence of gastro-duodenal ulceration [28].

In early studies involving *in vitro H. pylori* infection of gastric epithelial cells, proteins encoded by the cagPAI, with the exception of CagA, were shown to be required for IL-8 secretion and that IL-8 induction was regulated by the NF-kB pathway [9,10]. In addition, *H. pylori* peptidoglycans delivered through the type IV secretion system, identified by intracellular Nod1 receptor were also reported responsible for activating NF-kB leading to IL-8 induction [29]. It was later that CagA protein, was also recognized to contribute to the induction of IL-8 in gastric epithelial cells, through the activation of NF-kB pathway [30]. Others suggested that IL-8 activation was not dependent upon CagA phosphorylation on EPIYA-C motifs but on the highly conserved amino acid sequence FPLKRHDKVDDLSK termed as CRPIA [31], earlier identified as CagA multimerization (CM) motif [32] which has since been identified as a MARK2-protein kinase inhibitor (MKI) [33]. CagA has also been suggested to stimulate NF-kB induction and transcriptional activation of IL-8, through interaction and subsequent activation of TAK1 and this was mediated by TRAF6-Lys 63-ubiquitination [34]. However, *H. pylori*-induced proinflammatory responses remain a controversial issue, due to emerging contradictory reports [35]. Very recently CagL, has been suggested to induce secretion of interleukin-8 (IL-8) independently of CagA translocation and peptidoglycan-sensing nucleotide-binding oligomerization domain 1 (NOD1) signaling [36].

The aim of this study was to investigate CagA involvement in the secretion of IL-8 and more specifically to explore the molecular pathway by which phosphorylation on CagA terminal EPIYA-C motifs could potentially contribute to IL-8 gene induction. For this purpose, and in order to study CagA expression inside the epithelial cells through an infection, rather than transfection system, a series of *H. pylori* isogenic mutants based on P12 reference strain were produced, expressing CagA protein with varying numbers of phosphorylation-functional (EPIYA-C) and phosphorylation-deficient EPIFA motifs, while keeping the CM motifs intact. These strains were meticulously evaluated beforehand for their ability to adhere equally well on gastric epithelial cells, induce pilus formation and functionally translocate CagA protein inside epithelial cells [37]. Utilizing these strains we were able to show that CagA EPIYA-C phosphorylation is required for full transcriptional activation of IL-8 gene, irrespective of the number of these repeating units at the carboxyl-terminus of CagA. Moreover, our data suggest that CagA protein may contribute to NF-kB activation, potentially through interference with TAK1 and independently of ERK1/2 and AKT mediated NF-kB activation.

Materials and Methods

H. pylori strains

Based upon *H. pylori* P12 reference strain, which possesses CagA with an EPIYA-ABCC combination, a number of isogenic *H. pylori* mutants expressing CagA protein with a variable number of EPIYA-C motifs such as AB, ABC, ABCCC and the respective phosphorylation-deficient EPIFA mutants (Figure 1A and Figure 1B) were constructed . A CagA knock out (P12CagAKO) strain was constructed by interruption of *cagA* gene sequence following insertion of a chloramphenicol cassette [38]. A T4SS-defective *cagE* knock-out mutant of P12 (P12CagEKO) was kindly provided by Professor R. Haas. These strains were meticulously characterized in terms of growth rates and adhesion rates to AGS gastric epithelial cells (ATCC CRL 1739), as well as T4SS functionality, CagA expression and phosphorylation and the ability to induce scattering and elongation phenotype[37].

Culture of *H. pylori* and cell lines

H. pylori strains were cultured on Columbia Blood Agar (Oxoid) plates containing antibiotics, supplemented with 5% v/v horse blood and 1% v/v Vitox (Oxoid) under microaerophilic conditions (CampyGen, Oxoid) at 37°C, as described before [27].

AGS gastric epithelial cell line (ATCC CRL 1739) were cultured in 75 cm^2 flasks (Corning) in RPMI 1640 medium (Life Technologies) containing antibiotics (penicillin 10 U/ml, streptomycin 10 mg/ml) and supplemented with 10% FBS (Life Technologies).

$TAK1^{-/-}$ mouse embryonic fibroblasts (MEFs) and the respective control MEFs [39] were kindly provided by Professor S. Akira and were cultured as described in their publication.

In vitro infection and protein immunodetection

Gastric epithelial cell lines (AGS) were infected with *H. pylori* at MOI 100. Briefly, 4×10^5cells were seeded in 6-well plates and were left to adhere overnight. On the following day, two hours prior to infection, cells were washed with PBS (1x) and the medium was replaced with fresh, antibiotic free, RPMI 1640 containing 10%FBS. *H. pylori* strains were suspended in antibiotic free RPMI 1640 containing 10%FBS, adjusted to a concentration of approximately 10^8cfu/ml ($OD_{600nm} = 0.740$) and left for an hour to recover. Bacterial suspensions (100 μl) were used to infect gastric epithelial cells within 2 ml total volume.vAt collection times, total protein lysates were obtained in ice-cold lysis RIPA buffer as described before [27]. Equal protein amounts of lysates were separated by SDS-PAGE and transferred onto PVDF membranes (Immobilon P, Merck Millipore). Antibodies against

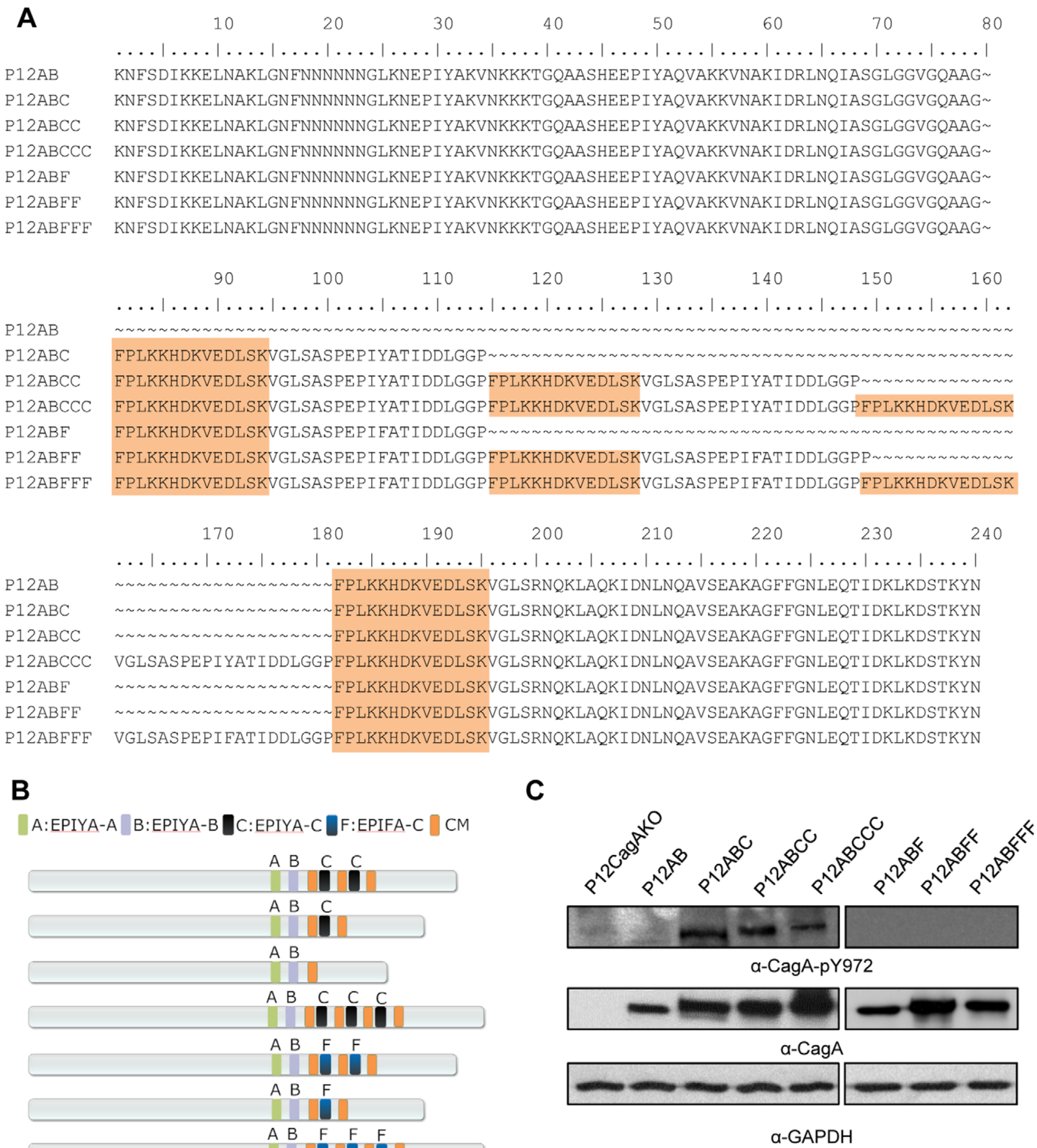

Figure 1. CagA EPIYA-C phosphorylation-functional and -defective *H. pylori* mutant strains. (A) Deduced CagA amino acid sequences, following nucleotide sequencing, depicting the EPIYA and EPIFA mutant motifs, as well as the MARK2-kinase inhibitor (CM) (shaded region). (B) Schematic representation of CagA protein expressed by the corresponding mutants. (C) Determination of CagA tyrosine phosphorylation by western blot utilizing α-CagA-pY972 antibody, which recognizes phosphorylated EPIYA-C motifs. CagA and GAPDH expression is also depicted, for control purposes.
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IKKa, phospho-IKBa (Ser32/36), phospho-ERK1/2 (Thr202/Tyr204), TAK1, phospho-TAK1 (Thr187), AKT, phospho-AKT (Ser473) (all purchased from Cell Signaling), CagA (polyclonal, Austral Biologicals) and GAPDH (Merck Millipore), were used according to suppliers' instructions. Specific antibody phospho-CagA (Tyr 972), which recognizes specifically phosphorylated

CagA at EPIYA-C motifs, was kindly provided by Professor S. Backert [40]. Quantity One software package (Bio-Rad) was utilized for band densitometry.

Immunoprecipitation

AGS cells (1×10^6) were seeded in 25 cm^2 flasks and were infected with *H. pylori* mutant strains at MOI 100. Following 1h of infection, cells were lysed by addition of 500 µl of NP40 lysis buffer containing protease and phosphatase inhibitors as described before [27]. Lysates were pre-cleared following incubation with 30 µl of Protein A-Sepharose from *Staphylococcus aureus* (Sigma). Respective immunoprecipitations were performed by addition of CagA polyclonal antibody (Austral Biologicals) or TAK1 monoclonal antibody (Cell Signaling) and consequent capture of the protein complex using 30 µl of Protein A-Sepharose. CagA detection by western blot was performed utilizing an anti-CagA monoclonal antibody supplied by Austral Biologicals or an anti-CagA monoclonal antibody raised against amino acids 1–300 supplied by Santa Cruz Biotechnology.

Quantification of IL-8 gene expression in *H. pylori* infected cells

Quantification of IL-8 gene expression relative to GAPDH, in *H. pylori*-infected (MOI 100) and uninfected AGS cells, was determined by the comparative Ct method [41]. RNA samples were collected at 2, 4 and 24 hours post infection in triplicates using the RNeasy Mini Kit (Qiagen). DNA sample contamination was eliminated with the use of RNase-Free DNase Set (Qiagen). cDNA was synthesized withvM-MLV (Promega) reverse transcriptase (80U) in a reaction that contained 20 mM Tris (pH 8.4), 50 mM KCl, 3.75 mM $MgCl_2$, 500 µM DNTPs, 0.4 µM, random hexamers primers (Life Technologies), 80 U RNase OUT Recombinant Ribonuclease Inhibitor (Life Technologies) and 22,2 µl RNA. Quantitative Real Time PCR amplification was carried out in a Stratagene Mx3005P QPCR System (Agilent Technologies), within 25 µl reaction mixtures of Platinum SYBR Green qPCR SuperMix (Life Technologies), 0.5 µM primers and 5 µl cDNA. Previously described PCR primers for human IL-8 [42], and GAPDH [43] were used. Three independent experiments were conducted involving three replicates for each individual sample time point.

Determination of IL-8 levels and inhibitors

Culture supernatants collected at selected time points from *H. pylori*-infected AGS cells (MOI 100), were collected following centrifugation at 13,000 rpm [27]. IL-8 levels were determined by commercial ELISA (eBioscience) according to manufacturer's protocol. Experiments in the presence of 5Z-7-Oxozeaenol (Merck) TAK1 specific inhibitor were carried out by addition of the inhibitor 2h prior to infection. Culture supernatants were collected at 4h post-infection. Initial experiments were conducted at a concentration range of 5–300 nM to determine the optimal concentration for TAK1 inhibition and then at 70nM thereof.

Results

Effect of EPIYA-C phosphorylation on IL-8 transcriptional activation and IL-8 secretion

In order to quantify potential effect of EPIYA-C phosphorylation on IL-8 induction we infected AGS cells with the whole range of mutant P12 strains, namely P12AB, P12ABC, P12ABCC (*wt*), P12ABCCC, P12ABF, P12ABFF, P12BFFF, P12CagEKO and P12CagAKO. Expression of CagA protein as well as EPIYA-C intracellular phosphorylation was observed at 2 hours post-infection (Figure 1C). IL-8 gene transcriptional activation was quantified at 2, 4, and 24 hours post-infection. Strains P12ABCCC, P12ABCC and P12ABC equally induced the highest levels of IL-8 transcription (120-, 120- and 112-fold respectively) compared to the uninfected control at 2 hours post-infection (Figure 2A). On the contrary, P12AB strain lacking the EPIYA-C phosphorylation sequences on CagA, induced IL-8 activation levels at 60-fold, a statistically significant reduction, compared to those induced by the strains P12ABCCC ($p = 0.006$), P12ABCC ($p = 0.007$) and P12ABC ($p = 0.002$). Moreover, IL-8 induced activation by the strains expressing CagA with phosphorylation-deficient EPIFA-C, was approximately halved for strains P12ABF and P12ABFF (64- and 70-fold) and even further reduced for the P12ABFFF (24-fold) strain at levels lower than those observed even for the P12CagAKO strain (42-fold). At 4 hours post-infection, IL-8 activation induced by the P12ABCCC strain was maintained at much higher level (50-fold) compared to those observed for P12ABCC ($p = 0.001$) and P12ABC strains ($p = 0.0009$, Figure 2A). These strains as well as P12CagAKO, P12ABFF and P12ABF induced background levels. Interestingly, IL-8 activation induced by P12AB strain remained unaltered between 2 and 4 hours, a result repeatedly obtained in over 5 repetitions of the experiment, for which we can offer no explanation. In contrast P12ABFFF strain contributed to a marginal increment between 2 and 4 hours ($p = 0.05$). At 24 hours post-infection IL-8 transcriptional activation in infected cells was at background levels for all strains (Figure 2A).

For the same experimental layout, we proceeded to determine IL-8 concentration in the supernatants of infected AGS gastric epithelial cell lines (Figure 2B). At 2 hours post-infection all strains induced similar amounts of IL-8 protein in the range of 600 pg/ml, with the exception of strains P12ABF or P12ABFF which exhibited significantly decreased levels (400 pg/ml) and P12ABFFF, for which levels of IL-8 protein were negligible and comparable to those observed for uninfected control cells (Figure 2B). Furthermore, P12ABFFF strain induced significantly lower levels of secreted IL-8 compared to all other strains, at 4 hours ($p = 0.001$) as well as 24 hours ($p = 0.004$) post-infection (Figure 2B). This characteristic delay in IL-8 secretion induced by strain P12ABFFF was apparent in all replicate experiments in which IL-8 levels secreted were found to be consistently lower than those observed even for the P12CagAKO strain. Similar results were obtained with another four independent P12ABFFF clones isolated during production (data not shown). At 24h all strains with functional EPIYA-C motifs (P12ABC, P12ABCC, P12ABCCC) induced approximately the same levels of IL-8 (Figure 2B), in any case significantly higher ($p = 0.011$) than those levels observed for the strain P12AB.

Collectively, these results suggest that phosphorylation of CagA in terminal EPIYA-C motifs contributes to IL-8 secretion by gastric epithelial cells. Most specifically, mutation of these EPIYA-C motifs, while keeping the CM motifs intact, leads to a characteristic delay in the initial transactivation of IL-8 gene, which consequently results to lower levels (approximately 2-fold reduction) of secreted IL-8, compared to the fully functional counterparts. Furthermore, secreted levels of IL-8 protein were observed not to be dependent upon the number of CagA EPIYA-C motifs, as similar levels were secreted by strains with one, two or three EPIYA-C motifs.

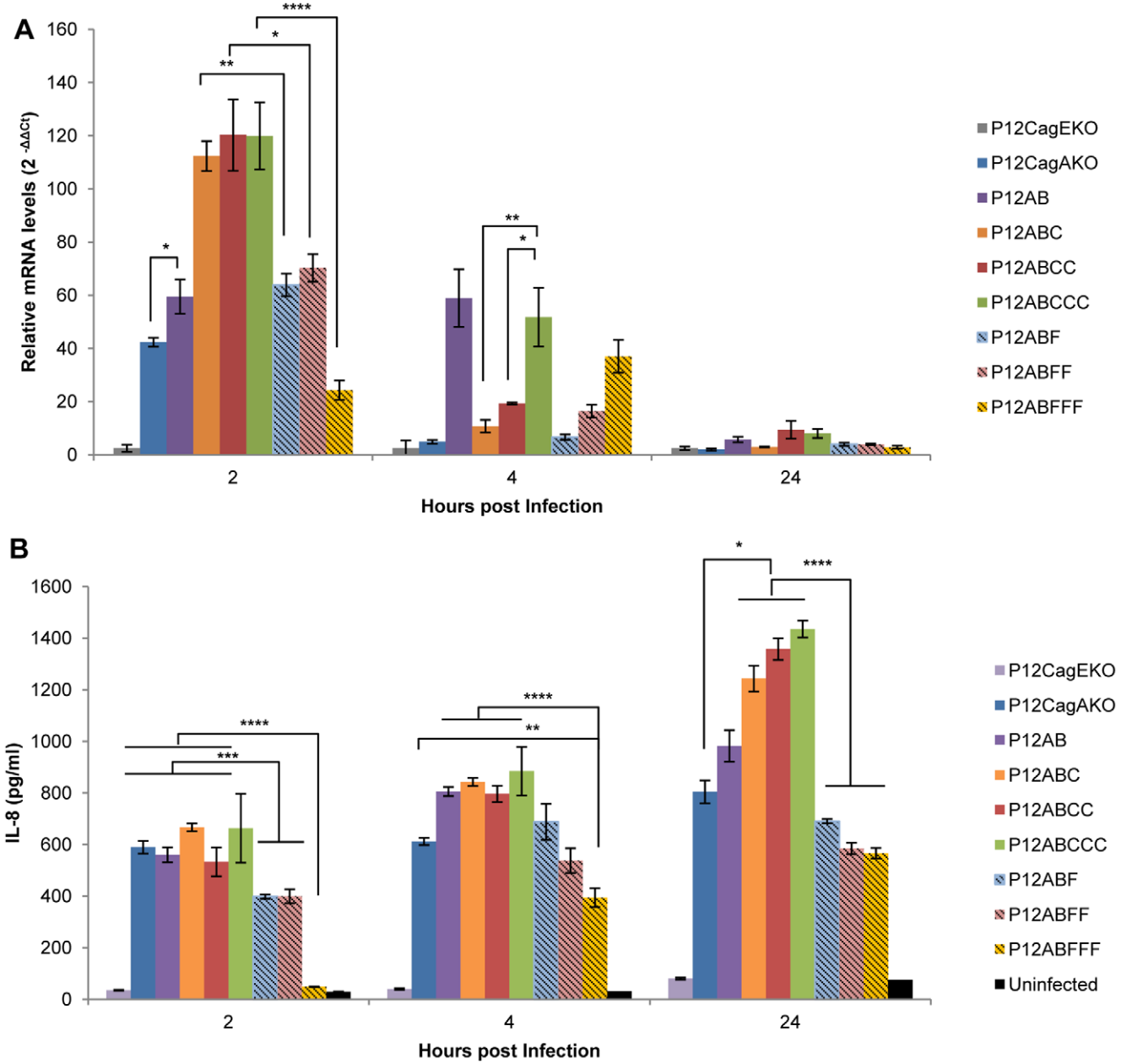

Figure 2. Effect of *H. pylori* infection on the induction and secretion of IL-8 by gastric epithelial cells. (A) Transcriptional activation of IL-8 gene in AGS cells, infected by the corresponding *H. pylori* mutant strains, expressing CagA with variable numbers of functional (EPIYA-C) or defective (EPIFA-C) phosphorylation motifs. IL-8 gene transcriptional activation was determined by a Quantitative Real Time PCR at 2, 4 and 24h post-infection. Statistical analysis was done by Student t-test and levels of significance depicted are *<0.05, **<0.01, ***<0.001, ****<0.0001. (B) IL-8 concentration levels determined by ELISA, in the supernatants of AGS cells, infected by *H. pylori* mutant strains. Statistical analysis and levels of significance as in (A).
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NF-kB activation triggers the IL-8 induction with respect to EPIYA phosphorylation

Induction of IL-8 following *H. pylori* infection of gastric epithelial cells has been attributed to NF-kB activation [9,10,30,34,44,45]. In our study, we monitored NF-kB activation by determination of the kinetics of the Ser32/36 phosphorylation of its inhibitor IkBα, leading it to proteasome degradation [23]. As transcriptional activation and production of IL-8 proved to be independent of the number of EPIYA-C motifs, we proceeded to conduct the experiments utilizing P12ABCCC and the respective P12ABFFF mutant, as well as P12AB and the P12CagAKO strains. Following infection of AGS cells over 24 hours, P12ABCCC strain induced the highest phosphorylation of IkBα amongst all strains, in a time-dependent manner (Figure 3). Most specifically, increased IkBα phosphorylation was evident as early as 15 min post-infection, reaching a peak at about 1–1,5 hours and decreasing thereof to reach background levels at 24 hours. Concomitantly, a marginal decrease in IkBα expression levels was evident during the early stages post-infection (15–90 min) compared to the late ones (2–24 hours). Similar induction kinetics of IkBα phosphorylation was observed for strains P12CagAKO and P12AB, although levels were lower than those observed for

P12ABCCC (Figure 3). On the contrary, strain P12ABFFF expressing CagA with phosphorylation-deficient motifs, exhibited the lowest IkBα phosphorylation levels while IkBα expression remained constant, throughout the experimental period (Figure 3). Collectively, these results illustrate that following infection of gastric epithelial cells with *H. pylori* strains, a higher activation of NF-kB is evident in the presence on phosphorylated EPIYA-C motifs. NF-kB is also activated, yet at considerably lower levels, in the absence of CagA expression or CagA phosphorylation and in the cases where EPIYA-C motifs are totally absent. Moreover, kinetics of IkBα phosphorylation (Figure 3) and hence NF-kB activation, appear to be in absolute agreement with the observed IL-8 transcriptional activation patterns (Figure 2A).

ERK1/2 and AKT contribution to the activation of NF-kB following phosphorylation of CagA on EPIYA motifs

ERK1/2 activation in the CagA-dependent induction of IL-8 through NF-kB activation has been illustrated before [30]. In our study, we have monitored the kinetics of ERK1/2 activation by determination of phosphorylation at Thr202/Tyr204 [46]. Our results showed that in cells infected by P12ABCCC, as well as the corresponding P12ABFFF phosphorylation-deficient mutant, ERK1/2 activation occurred at similar significantly higher levels compared to strains P12AB and P12CagAKO, during the initial stages of infection (mean difference at 30 min of 1.97 and 95%CI 0.80 to 3.14 [$P=0.006$], mean difference at 60 min of 1.06 and 95%CI of 0.27 to 1.84 [$P=0.004$], mean difference at 90 min of 1.140 and 95%CI of 0.48 to 1.79 [$P=0.016$]), suggesting that ERK1/2 activation is EPIYA-C motif phosphorylation-independent (Figure 4A and Figure 4B). On the contrary, strains P12AB and P12CagAKO induced similar significantly higher ERK1/2 activation levels much later, after the initial 90 min post-infection (mean difference at 2 hours of 1.80 and 95%CI of 0.83 to 2.77 [$P=0.005$], mean difference at 4 hours of 1.03 and 95%CI -0.13 to 2.18 [$P=0.004$]), suggesting that early ERK1/2 activation may depend upon the presence of sequences surrounding the EPIYA-C motifs. Recently, the CagA protein conserved amino acid sequence CRPIA was suggested to play a significant role in the activation of NF-kB via AKT kinase [31]. Our observations with regards to potential involvement of AKT in the activation of NF-kB suggest that the activation pattern of AKT does not match with this of NF-kB activation. More specifically, following *H. pylori* infection of AGS cells, we determined that activation of AKT Ser473-phosphorylation is independent of CagA EPIYA-C motif status (Figure 4D).

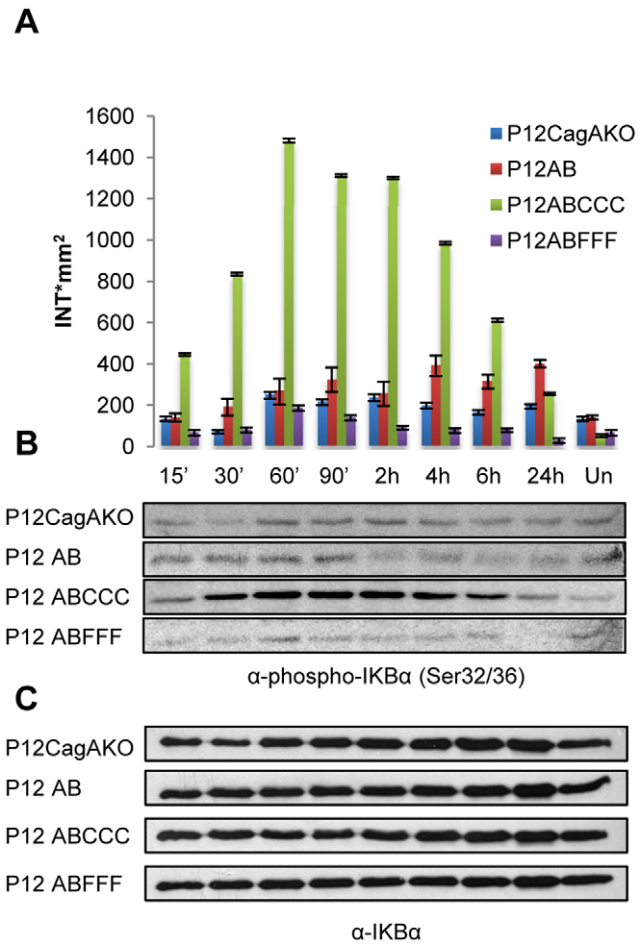

Figure 3. NF-kB activation in AGS cells following *H. pylori* infection. IkBα phosphorylation at Ser32/36 in AGS cells infected with *H. pylori* mutants, expressing CagA with phosphorylation-functional (EPIYA-C) or -defective (EPIFA-C) motifs. (A) Quantification of IkBα phosphorylation determined by band densitometry utilizing Quantity One software package. (B) Phosphorylation of IkBα and (C) Expression of total IkBα. Un: uninfected cells.
doi:10.1371/journal.pone.0056291.g003

Activation of NF-kB possibly through TAK1 activation following CagA EPIYA-C phosphorylation*v*

Recently, TAK1 was reported to be involved in *H. pylori*-induced NF-kB activation following its interaction with CagA protein and TRAF6 [34]. More specifically, it was suggested that CagA physically associates with TAK1 in vitro and in vivo and thereby enhances TAK1 activation which also requires TRAF6-mediated Lys63 ubiquitination. Furthermore, another study suggested that phosphorylated CagA can lead to activation of NF-kB and consequently transcriptional activation of IL-8, through p38 phosphorylation [47], whose upstream regulator is TAK1 [48,49].

To confirm TAK1 involvement, we infected AGS cells with our CagA phosphorylation-functional and -deficient mutants in the presence of 5Z-7-Oxozeaenol which is reported to be a specific inhibitor of TAK1 in a number of studies [50–54] and determined the levels of secreted IL-8 in the culture supernatants (Figure 5A and Figure 5B), at 4h post infection, due to the labile nature of the inhibitor. Utilizing the P12ABCCC strain to infect AGS cells we observed that in the presence of TAK1 inhibitor IL-8 production was abrogated in a concentration-dependent manner (Figure 5A). Moreover, in the presence of 70 nM 5Z-7-Oxozeaenol we observed a complete arrest of IL-8 production by AGS cells infected with the whole range of CagA phosphorylation-functional as well as phosphorylation-deficient strains, P12CagAKO and the P12CagEKO (Figure 5B). In order to further clarify TAK1 involvement in *H. pylori* induced NF-kB activation we infected TAK1$^{-/-}$ mouse embryonic fibroblasts (MEFs) and the respective normal control MEFs with P12ABCCC, P12ABFFF, P12AB, P12CagAKO and P12CagEKO mutants. We observed NF-kB activation following *H. pylori* infection, only in the presence of TAK1 expression in control MEFs and not in the TAK1$^{-/-}$ cells (Figure 5C). As expected, unlike P12CagAKO which contributed to NF-kB activation in control MEFs, P12CagEKO did not induce NF-kB activation either in control or TAK1$^{-/-}$ MEFs. Collectively these results suggest that NF-kB-dependent IL-8 induction in *H. pylori* infected gastric epithelial cells may possibly be mediated altogether through TAK1, irrespective of the CagA protein status.

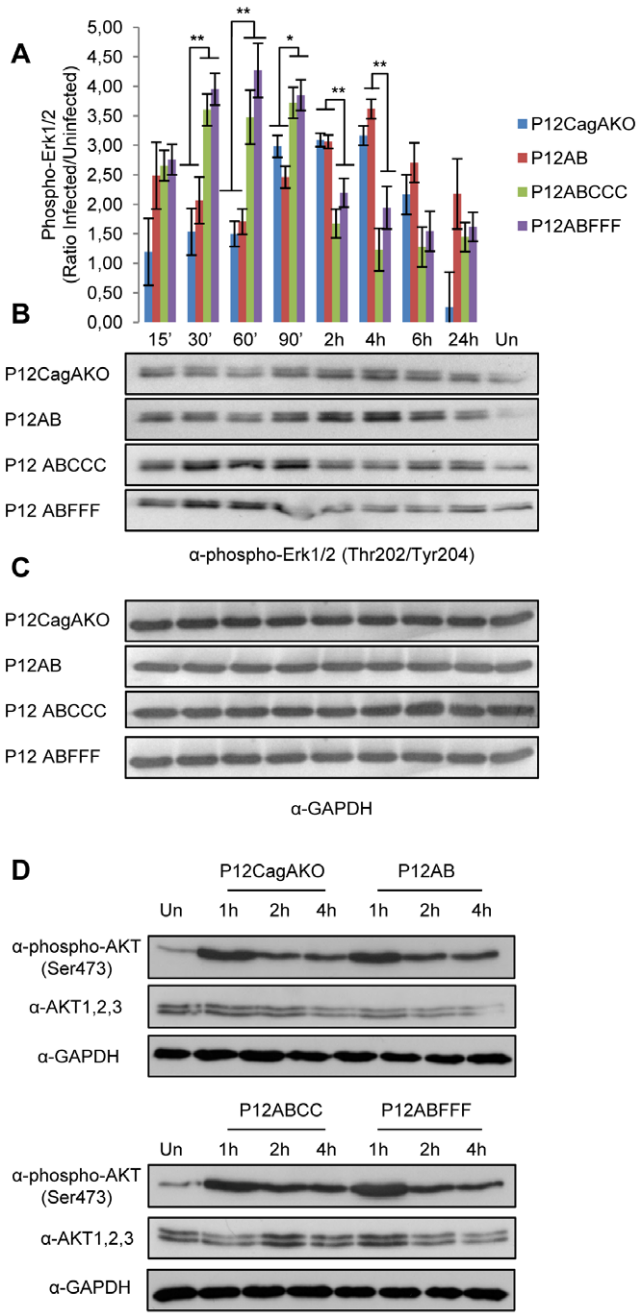

Figure 4. ERK1/2 and AKT activation in AGS cells upon infection with *H. pylori*mutant strains expressing CagA with phosphorylation-functional (EPIYA-C) or -defective (EPIFA-C) motifs. (A) Quantification of ERK1/2 phosphorylation at Thr202/Tyr204 by band densitometry in two independent experiments is depicted by plotting phospho-ERK1/2 (ratio infected to uninfected cells) for each time point.vStatistical analysis was done by Student t-test and levels of significance depicted are *<0.05 and **<0.01. (B) Representative ERK1/2 phosphorylation at Thr202/Tyr204 and (C) the corresponding GAPDH expression. (D) Phosphorylation of AKT at Ser473 determined in total protein lysates from AGS cells infected with corresponding AKT1, 2, 3 and GAPDH expression. Un: uninfected cells.
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We proceeded to confirm by immunoprecipitation earlier observations which suggest that CagA may interact with TAK1 [34], in total lysates of AGS cells infected with the P12ABCCC, P12ABFFF and P12AB strains. We were able to identify TAK1 (Figure 6A) in anti-CagA immunoprecipitated protein complexes in total lysates of AGS cells infected with all three strains (P12ABCCC, P12ABFFF and P12AB).vEqually we were able to detect CagA proteins utilizing a C-terminal specific CagA antibody (Figure 6B), as well as, an N-terminal specific anti-CagA antibody (Figure 6C) within the monoclonal TAK1 antibody-immunoprecipitated protein complex. Our results suggest that CagA may participate in the same protein complex along with TAK1, irrespective of the presence or the phosphorylation status of EPIYA-C motifs in CagA. We also observed that following α-TAK1 immunoprecipitation, the α-CagA monoclonal antibody raised against the C-terminal (Austral Biologicals) failed to detect the P12AB CagA variant (Figure 6B) possibly because it maps exactly on the EPIYA-C domains. On the contrary, utilizing an N-terminal specific anti-CagA antibody (Figure 6C) we were able to detect CagA, suggesting that sequences following immediately after the EPIYA-C motif, such as the CM motif present in the P12AB CagA protein (Figure 1A and Figure 1B) may also possibly be implicated in the CagA-TAK1 interaction. This motif has been suggested to be a putative kinase anchor [33], however, further work is required to demonstrate this in our case. Finally, in order to clarify potential role of CagA protein EPIYA-C phosphorylation on TAK1 activation, we attempted to determine TAK1 phosphorylation on Thr187 as well as TAK1 protein expression in *H. pylori* infected AGS cells. However, we detected a very marginal increase in pThr187-TAK1 in P12ABCCC strain infected AGS cells, at 60 min post infection, compared to that observed in uninfected cells, followed by a decrease thereof (Figure S1A and S1B). Similar results were also observed for the P12ABFFF and P12AB strain-infected AGS cells, delayed for two hours, whereas no such TAK1 activation could be detected for cells infected with P12CagAKO strain. Such weak observations have been described before [34], with an equally weak signal for activated TAK1, a problem that is also presented in other studies [55].

Discussion

H. pylori induces activation of a complex cytokine and chemokine network in the gastric mucosa and different bacterial and environmental factors, as well as host genetics seem to dictate the balance between tolerance and pro-inflammatory response in the course of *H. pylori* infection. CagA protein has been suggested to manifest its pathogenic role through EPIYA-dependent and EPIYA-independent interactions, in a number of intracellular signaling pathways, regulating cell motility and elongation, disruption of intercellular junctions and cellular polarity, as well as proliferation and inflammation mechanisms invoked by gastric epithelial cells [11]. Moreover, since most of these pathways have been implicated in the development of cellular malignancy there is increasing support that CagA protein, under persistent *H. pylori* infection may function as a bacterial oncoprotein [56,57]. Presence of chronic active inflammatory response in the lamina propria is the hallmark of *H. pylori* infection and the main regulators of this inflammatory response are chemokines. *H. pylori* stimulates the gastric epithelium to produce IL-8, a potent neutrophil chemokine [5] and its activity was shown to correlate to histological severity in cases of *H. pylori*-induced antral gastritis [7]. Although early reports suggested that cagPAI functionality was a prerequisite for IL-8 induction and secretion by gastric epithelial cells [58], later studies proved beyond doubt, that this phenomenon was also potentiated by the expression of CagA protein [30]. Moreover, others suggested that IL-8 activation was not dependent upon the phosphorylation on EPIYA-C motifs but

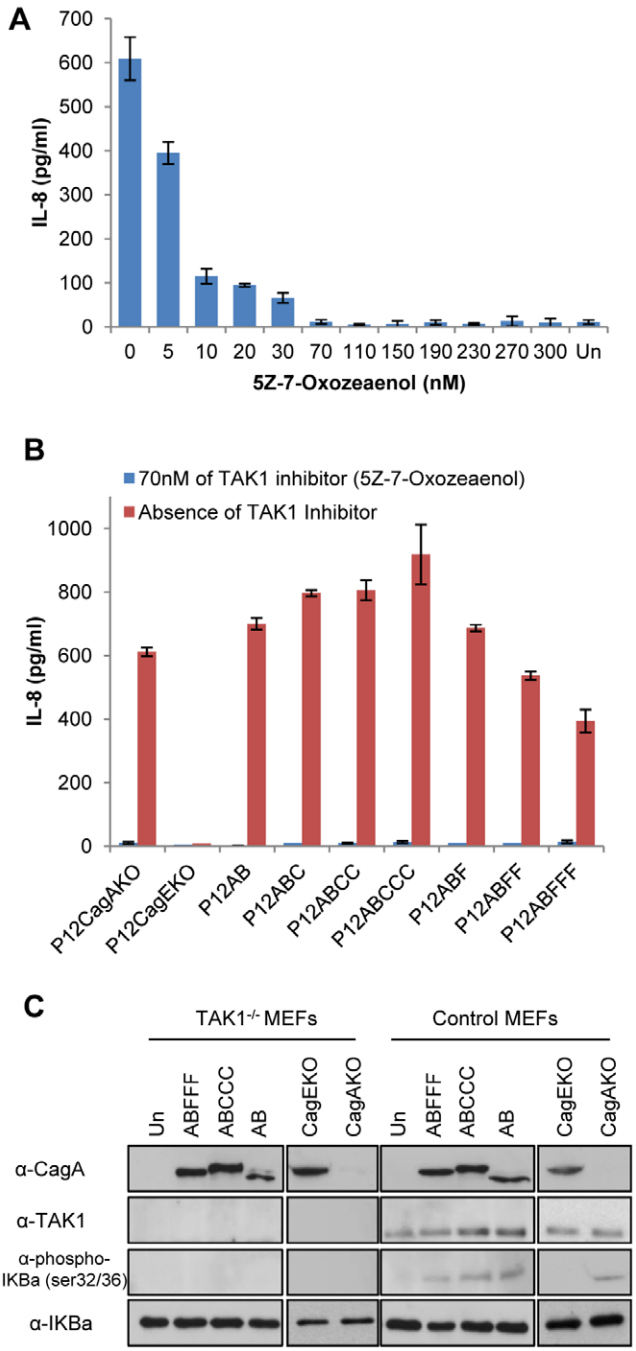

Figure 5. Potential involvement of TAK1 in IL-8 secretion following *H. pylori* infection. (A) Dose-dependent inhibition of IL-8 secretion, in the presence of the specific TAK1 inhibitor 5Z-7-Oxozeaenol (5–300 nM), following 4 hour infection of AGS cells with *H. pylori* P12ABCCC strain. (B) IL-8 secretion in the supernatants of AGS cells following 4 hour infection by the corresponding *H. pylori* CagA mutants, in the absence or presence of 70 nM 5Z-7-Oxozeaenol. (C) Expression of CagA, TAK1, IKBa phosphorylation at Ser32/36 and total IkBα determined by western blot, in total lysates of TAK1$^{-/-}$ mouse embryonic fibroblasts (MEFs) and respective control MEFs infected with *H. pylori* mutant strains for 4 hours. Un: uninfected MEFs.
doi:10.1371/journal.pone.0056291.g005

on a highly conserved amino acid sequence around EPIYA termed as CRPIA [31], also recognized to be a CagA multimerization motif [32], implicated in kinase anchoring and inhibition [33]. Very recently, *H. pylori* has been shown to induce IL-8 via interaction of its T4SS constituent CagL with the host receptor integrin b1 and the subsequent activation of MAPKs and NF-kB [36]. In this study we report that CagA phosphorylation on EPIYA-C motifs contributes to the induction of IL-8 gene in gastric epithelial cells, through activation of the NF-kB pathway, possibly via TAK1 interaction. Given the potential diversity of clinical strains, we utilized isogenic *H. pylori* CagA mutants based on the reference *H. pylori* strain P12, expressing CagA protein with variable numbers of EPIYA-C phosphorylation motifs (P12AB, P12ABC, P12ABCC, P12ABCCC) and their respective EPIFA-C phosphorylation deficient counterparts (P12ABF, P12ABFF, P12ABFFF), while maintaining intact the EPIYA-A and B motifs[37]. We carefully screened our *H. pylori* mutant strains with regards to growth rates, adhesion rates to gastric epithelial cells and for polar effects to other cagPAI genes, pilus formation, type IV secretion system functionality, as well as CagA expression and phosphorylation[37]. We demonstrated that induction of the scattering and elongation phenotype, was as expected, dependent upon the number of motifs and phosphorylation status of the EPIYA-C motifs[37]. A number of previous reports have been based upon observations from transfected CagA-expressing species which, although undoubtedly very valuable for the study of bacterial virulence factors, may lead to erroneous results due to potentially ectopic over-expression of the bacterial protein under study. Furthermore, confusing results due to individual strain genetic variability, have also been attributed to the use of clinical isolates expressing CagA with differential number of EPIYA-C motifs [35]. In our study, IL-8 induction and secreted levels do not appear to be dependent upon the number of EPIYA-C repeats within CagA, as all our strains with functional EPIYA-C motifs were capable of inducing the same levels of IL-8 transcriptional activity and chemokine. These results on IL-8 secretion, are in line with previous observations reported by our group utilizing 4 different pairs of isogenic clinical strains isolated from the same patient, expressing CagA with variable numbers of EPIYA-C motifs [27,28]. In the same studies, CagA-positive clinical isolates with higher numbers of EPIYA-C motifs were not found to be associated with the severity of inflammatory response in the patient lamina propria. On the other hand, presence of phosphorylation-functional EPIYA-C motifs seems to contribute significantly to the *cagA*-dependent IL-8 induction, because deletion of these motifs, as in the case of P12AB, or mutation to the phospho-deficient EPIFA, in P12ABF and P12ABFF strains, dramatically reduced the levels of IL-8 induction. Most intriguingly, all P12ABFFF clones showed a characteristic delay in early IL-8 induction compared to all the other mutants, consistent of a potential transient inhibition of the T4SS, although earlier work has proven successful pilus formation and CagA translocation[37]. Alternatively, such an inhibition in IL-8 induction by the P12ABFFF strains may be associated with the presence within the CagA carboxyl-terminal region of four repeats of the putative kinase inhibitor motif FPLKRHDKVDDLSK [33], in the absence of any EPIYA-C functional domains, which dictate the hierarchic phosphorylation-dependent CagA activity [24].

In addition to the EPIYA-C effect, our data suggest that presence of EPIYA-A and EPIYA-B may marginally also contribute to IL-8 induction, because the CagA P12AB strain was able to induce significantly higher levels of IL-8 transcriptional activation compared to the P12CagAKO, yet similar to those observed following infection with the P12ABF and ABFF strains. This may also suggest that the number of CM does not seem to play a role in the induction of IL-8, as AB, ABF and ABFF have 1, 2 or 3 such motifs, respectively, and they all induce equal levels of

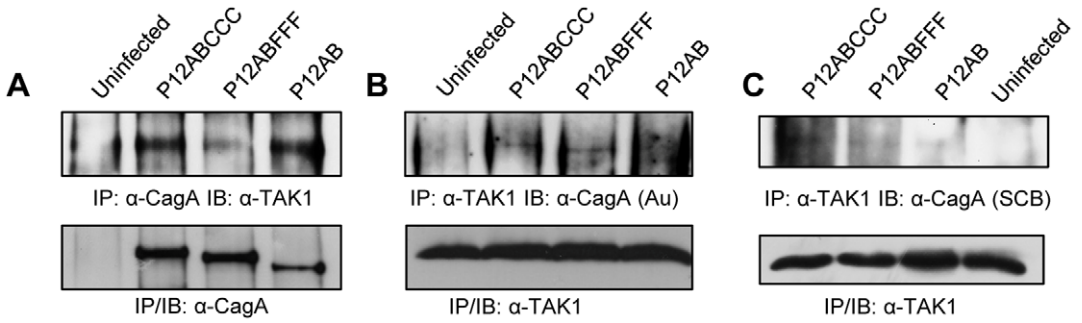

Figure 6. Immuno-detection of TAK1 and CagA in α-CagA or α-TAK1 respective immuno-precipitated lysates derived from AGS cells infected with *H. pylori* CagA mutant strains, at 1 hour post infection. (Panel A) Immunoprecipitation utilizing polyclonal α-CagA antibody (Austral Biologicals) and TAK1 western blot immunodetection utilizing monoclonal α-TAK1 antibody. Immunoprecipitation utilizing monoclonal α-TAK1 antibody and CagA immunodetection utilizing an α-CagA monoclonal antibody raised against the C-terminal (Panel B) or the N-terminal (Panel C) end of CagA protein. Note that the α-CagA monoclonal antibody raised against the C-terminal (Austral Biologicals) maps exactly on the EPIYA-C domains and fails to detect the P12AB CagA variant in panel B.
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IL-8 transcription at 2 hours, as well as NF-kB activation (data not shown). Furthermore, our results following infection with the P12CagAKO strain, are in line with observations suggesting *cagA*-independent IL-8 gene transcriptional activation and secretion, following recognition of peptidoglycans, by the intracellular Nod1 receptor [29] or *H. pylori* lipopolysaccharide by TLRs [45,59] or CagL binding to integrin b1 [36]. In any case, our data suggest that *cagA*-dependent contribution through EPIYA-C phosphorylation may be equally significant for full activation of IL-8 transcriptional activity.

Another aim of this work was to shed light on the molecular pathway by which NF-kB is activated following EPIYA-C phosphorylation. Until recently, activation of NF-kB following *H. pylori* infection was shown to be a result of ERK and AKT activation [14,30,31]. Here we provide evidence that activation of ERK and AKT during the early stages post-infection may be influenced by the presence of EPIYA-C motifs, albeit in a phosphorylation-independent manner. In addition, our data suggest that activation of ERK and AKT does not exactly coincide to NF-kB activation. More specifically, we observed that in the presence of sequences surrounding EPIYA-C motifs, as in the case of P12ABCCC and P12ABFFF strains, induction of ERK1/2 was evident at early stages as quickly as 30' post-infection irrespective of EPIYA phosphorylation. On the contrary, in the absence of EPIYA-C motifs as in the P12AB and P12CagAKO strains, ERK1/2 activation was evident at much later stages post-infection (2 - 4h). In the case of AKT, we observed a CagA-independent effect of *H. pylori* infection on AKT phosphorylation at Ser473. That is most likely consistent with its contribution to the control of survival and apoptosis, rather than that of other signaling pathways [60]. Further work needs to be done in order to understand how phosphorylation of CagA in EPIYA-C motifs may contribute to the activation of these two key molecules.

Finally, our data suggest that TAK1 may indeed play a critical central role in the activation of IL-8, because infection of gastric epithelial cells with all our *H. pylori* CagA mutants, in the presence of a TAK1 specific inhibitor [50-54], totally abrogated IL-8 secretion, even in the case of the P12CagAKO. Identical results were obtained when we infected $TAK1^{-/-}$ mouse embryonic fibroblasts with our *H. pylori* CagA mutant strains including the CagA negative mutant and observed NF-kB activation only in the presence of TAK1 expression in control MEFs and not in the $TAK1^{-/-}$ cells (Figure 5C). This suggests that TAK1 may be the converging molecule playing a central role for NF-kB activation during *H. pylori* infection, involving as well pathways activated by TLRs or NOD1 receptors as recently proposed [13]. Moreover, our data suggest that in the case of CagA-positive strains, TAK1 and CagA may participate in the same protein complex, confirming earlier observations showing that CagA can directly interact with TAK1 and TRAF6 [34]. Our results suggest that this TAK1-CagA interaction is independent of EPIYA-C-phosphorylation and may involve the CM motif preceding the EPIYA-C motif. Nevertheless, a whole range of experiments involving protein-protein interactions and mutational analysis needs to be performed in order to further dissect this interaction. We speculate that within the first hour post-infection, TAK1 may be recruited on CagA irrespective of EPIYA-C phosphorylation, however its consequent activation may depend upon EPIYA-C phosphorylation by c-Src and therefore TAK1 may be activated much faster in the presence of CagA-positive compared to CagA-negative strains. A possible explanation is that the different types of EPIYA phosphorylation motifs become phosphorylated by cellular kinases Abl and Src, at different time points following the delivery of CagA by the type IV secretion system. Indeed, EPIYA-C motifs are the first to be phosphorylated by Src, followed by Abl phosphorylation of EPIYA-A or EPIYA-B motifs [24], although more work is needed in order to explore this phenomenon with strains expressing CagA with multiple EPIYA-C motifs. Our results however, failed to show convincing TAK1 activation in the presence of functional EPIYA-C motifs in CagA compared to other CagA species examined in this study and further investigation is needed in order to prove this argument.

In conclusion, presence of functional EPIYA-C motifs in CagA protein, seem to contribute significantly in the transcriptional activation of IL-8, through NF-kB activation. This effect looks to be independent from *H. pylori*-dependent activation of AKT and ERK and may involve TAK1. This is the first systematic investigation of the role of EPIYA-C motifs in CagA in an isogenic background. Previous reports investigating their role in inflammation and disease outcome are based on comparison of different isolates. These comparisons however, suffer from the fact that *H. pylori* is genetically variable and has other phase-variable and mosaic genes. On the contrary, this is a systematic investigation and does not suffer from the aforementioned problems.

Supporting Information

Figure S1 TAK1 activation following infection of AGS cells with *H. pylori* CagA mutant strains. (A) Quantification of TAK1 phosphorylation at Thr187 by band densitometry. (B) Expression of phospho-TAK1 (Thr187) in total protein lysates and corresponding (C) TAK1 protein expression and (D) control GAPDH expression in total protein lysates from AGS cells infected with *H. pylori* CagA mutant strains as indicated.
(TIF)

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Author Contributions

Conceived and designed the experiments: KSP DNS. Performed the experiments: KSP ISS. Analyzed the data: KSP ISS DNS. Contributed reagents/materials/analysis tools: EH AFM. Wrote the paper: KSP ISS AFM EH DNS.

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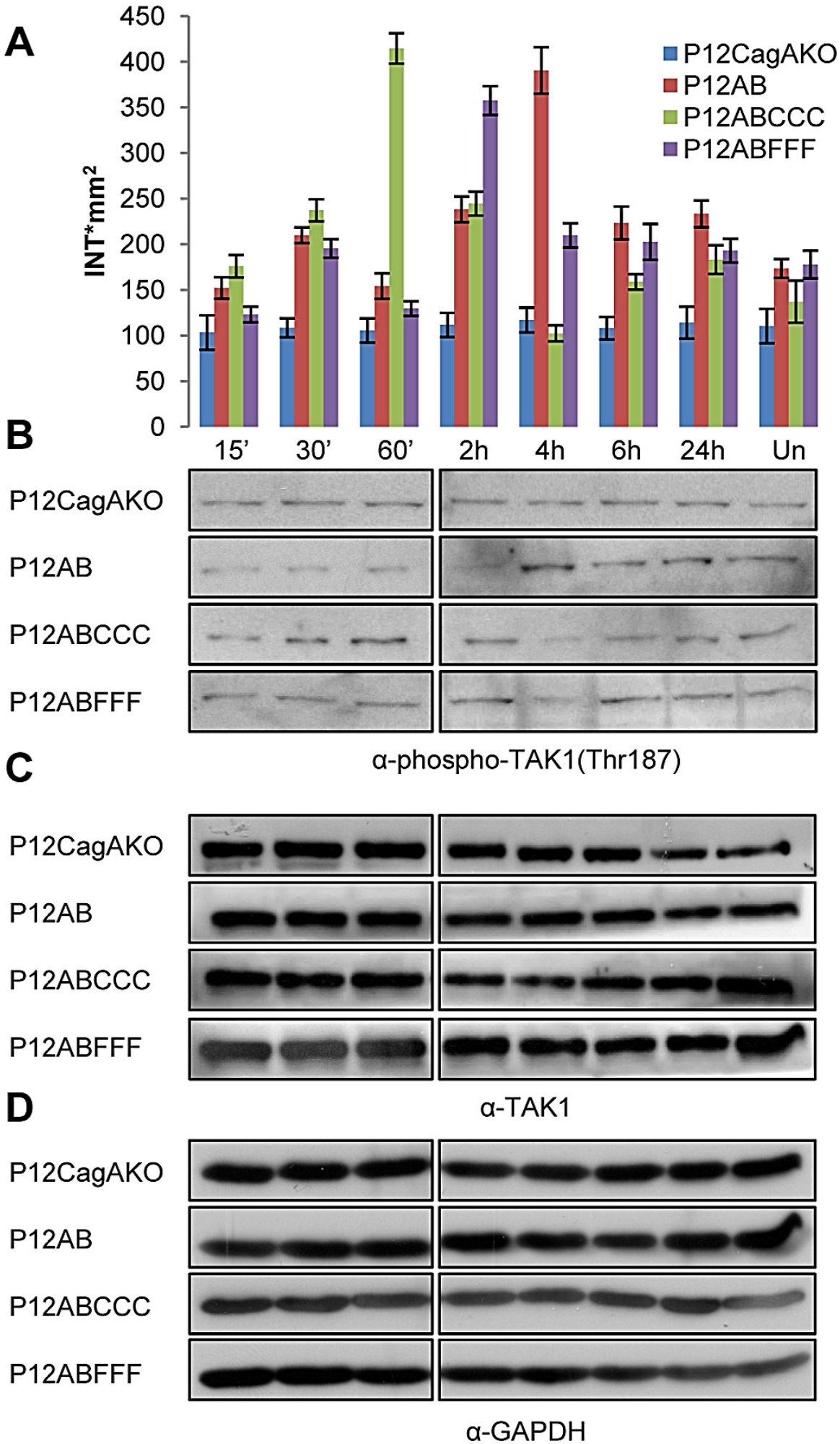
Figure S1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Helicobacter pylori infection in patients with nonalcoholic fatty liver disease

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ABSTRACT

Objective. Clinical data regarding *Helicobacter pylori* (*Hp*) infection in nonalcoholic fatty liver disease (NAFLD) are limited. The aim was the evaluation of *Hp* infection in patients with NAFLD and its association with disease severity.

Methods. 28 patients with biopsy-proven NAFLD (15 with simple nonalcoholic fatty liver [NAFL], 13 with nonalcoholic steatohepatitis [NASH]) and 25 matched healthy controls were recruited. Blood samples for anti-*Hp* Immunoglobulin G (IgG) and standard biochemical tests were obtained after overnight fasting, and ^{13}C urea breath test was performed before liver biopsy in NAFLD group.

Results. Higher rates of anti-*Hp* IgG ($P = .038$) were observed in NAFLD compared to control group. Only two NAFLD patients neither were *Hp* IgG seropositive nor did they have a history of eradication treatment compared to 11 control subjects ($P = .002$). Both *Hp* infection (assessed by history of *Hp* eradication treatment and/or *Hp* IgG seropositivity) ($P = .034$) and log(HOMA-IR) ($P = .007$) could independently predict NAFLD in logistic regression analysis. There were similar rates of *Hp* IgG seropositivity or positivity in ^{13}C urea breath test or their combination between NAFL and NASH patients. There were no significant differences in steatosis grade, fibrosis stage, lobular or portal inflammation, or ballooning, when NAFLD patients were divided according to *Hp* IgG seropositivity or ^{13}C urea breath test positivity.

Conclusions. *Hp* infection may represent one more hit contributing to the pathogenesis of NAFL, though not to the progression from NAFL to NASH. These results warrant further validation. If confirmed, eradicating *Hp* infection may have certain therapeutic perspectives in NAFLD treatment.

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; CV, coefficient of variation; GGT, gamma-glutamyl transferase; HMW, high molecular weight; HOMA-IR, homeostatic model of assessment - insulin resistance; *Hp*, *Helicobacter pylori*; hsCRP, high sensitivity C-reactive protein; IgG, immunoglobulin G; IR, insulin resistance; LDL-C, low-density lipoprotein cholesterol; NAFL, simple nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD Activity Score; NASH, nonalcoholic steatohepatitis; T2DM, type 2 diabetes mellitus; TNF, tumor necrosis factor.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is considered to be the hepatic manifestation of insulin resistance (IR) syndrome [1], given that IR plays a key role in its pathogenesis [2]. NAFLD ranges from simple nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH), whose features are steatosis, inflammation and fibrosis; advanced stages of NASH may ultimately result in liver cirrhosis, subacute liver failure and hepatocellular carcinoma [2]. The prevalence of IR syndrome and its related morbidity are rapidly increasing worldwide [2]. Apart from the usual risk factors, such as sedentary lifestyle and dietary habits, exposure to other factors, including endocrine disruptors [3] and *Helicobacter pylori (Hp)* infection [4], has been recently proposed in an attempt to elucidate the multi-faceted aspects of the pathogenesis of IR.

Although evidence is not always conclusive, *Hp* infection has been implicated in a variety of extradigestive conditions, including cardiovascular, lung, hematologic, ophthalmic, skin, neurologic and hepatobiliary diseases [5,6]. Regarding hepatobiliary diseases, *Hp* is thought to have deleterious consequences on the hepatobiliary tract, because of its proximity to the stomach: the liver may be damaged by *Hp* toxins and constituents circulating in blood coming out from the gastroduodenal area and the biliary epithelium can easily be colonized by bacteria from the duodenum [5,7]. Regarding IR-related morbidity, *Hp* infection has been implicated in the pathogenesis of obesity and type 2 diabetes mellitus (T2DM) [4–6]. However, only limited clinical data suggest that *Hp* infection might be a risk factor for the development of NAFLD [8–10]. The primary endpoint of this study was the evaluation of *Hp* infection in patients with biopsy-proven NAFLD and its association with the disease severity.

2. Methods

This was a single-center, cross-sectional study. Patients with NAFLD and controls were recruited on an outpatient basis between June 2008 and November 2010. Determination of eligibility was based on medical history, physical examination, and standard tests (serum aspartate transaminase [AST], alanine transaminase [ALT], gamma-glutamyl transferase [GGT], total alkaline phosphatase, total and indirect bilirubin) and procedures (liver ultrasound imaging) performed during the screening visit. All participants provided an informed consent. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local ethics committee. Inclusion criteria for the NAFLD patients were: 1) age ≥18 years; 2) bright liver on ultrasound imaging and increased liver function tests for at least 6 months before liver biopsy; and 3) patient's consent for liver biopsy. Age- gender- and body mass index (BMI)-matched individuals were recruited for control group. The control group consisted mainly of apparently healthy individuals subjected to regular check-up for professional needs. Inclusion criteria for the controls were: 1) age ≥18 years; 2) normal liver ultrasound imaging; and 3) normal liver function tests. Controls did not undergo a liver biopsy, because of obvious ethical considerations.

Exclusion criteria for both NAFLD patients and controls were: 1) ethanol consumption more than 20 g/day; 2) liver cirrhosis; 3) other liver disease (viral hepatitis, autoimmune hepatitis, primary sclerosing cholangitis, primary biliary cirrhosis and overlap syndromes, drug-induced liver disease, hemochromatosis, Wilson's disease, α1-antitrypsin deficiency); 4) type I diabetes mellitus; 5) pancreatitis; 6) uncontrolled hypothyroidism or hyperthyroidism; 7) adrenal insufficiency; 8) renal failure; 9) thrombotic disorders; 10) cancer; 11) pregnancy; 12) addiction to any drug; 13) use of the following medications within a 12-month period before screening: estrogens, progestins, glucocorticosteroids, thiazolidinediones, insulin, sibutramine, orlistat, rimonabant, vitamin E, vitamin C, ursodeoxycholic acid, ferrum sulfate, interferon, tamoxifen, amiodarone, biologic agents, folate or vitamin B supplements, antibiotic, any medication against tuberculosis, epilepsy or viruses, or any medication affecting hemostasis, such as antiplatelet agents, aspirin or oral anticoagulants; and 14) use of intravenous glucose administration or parenteral nutrition within a 1-month period before screening. Patients with T2DM not being on thiazolidinediones or insulin were eligible for this study.

Morning (8–9 am) fasting serum samples were collected, and fasting ^{13}C urea breath test was also performed 1–2 h prior to liver biopsy, which was performed under computed tomography-guidance by an experienced radiologist (EZ) and interpreted by two experienced pathologists (KP, EK). Serum AST, ALT, GGT and glucose were measured within 1 h after blood drawing, with standard methods using an automated analyzer (Olympus AU2700; Olympus, Hamburg, Germany). Sera were also immediately frozen at −30 °C for the measurement of insulin, high sensitivity C-reactive protein (hsCRP), anti-*Hp* Immunoglobulin G (IgG), tumor necrosis factor (TNF)-α, total and high molecular weight (HMW) adiponectin. Insulin was measured with two-site immunochemiluminescence on a Immulite 2500 immunoassay system (Siemens Healthcare Diagnostics, Deerfield, IL; intra-assay coefficient of variation [CV] 3.3%–5.5%, total CV 4.1%–7.3%). hsCRP was measured with latex-enhanced immunonephelometric assay on a BNII analyzer (Siemens Healthcare Diagnostics, Deerfield, IL, USA; total CV 4.0%–5.0%). Anti-*Hp* IgG, TNF-α, total and HMW adiponectin were measured with enzyme-linked immunosorbent assay (ELISA) on a ELx800 Absorbance Microplate Reader automated analyzer (BioTek, Winooski, VT, USA), by using the following commercial kits, respectively: anti-*Hp* IgG Quantitative ELISA kit (Phoenix Europe, Karlsruhe, Germany; intra-assay CV 6.4%–9.1%, inter-assay CV 7.5%–10.5%); TNF-α human ELISA kit (R&D Systems, Minneapolis, MN, USA; intra-assay CV 4.2%–5.2%, inter-assay CV 4.6%–7.4%), adiponectin human ELISA kit (Phoenix Europe, Karlsruhe, Germany; intra-assay CV 5.0%, inter-assay CV 6.0%); adiponectin multimeric ELISA kit (ALPCO Immunoassays, Salem, NH, USA; intra-assay CV 3.3%–5.0%, inter-assay CV 5.7%). ^{13}C urea breath test was performed by *Hp* test INFAI (INFAI, Bochum, Germany) and the analysis was carried out using an isotope ratio mass spectrometer (Faran Laboratories, Athens, Greece). Anti-*Hp* IgG levels > 10 U/mL were considered positive. Delta ^{13}C

(between T_0 min and T_{30} min) > 4.0 per mill at ^{13}C urea breath test was considered positive.

BMI was calculated by the formula body weight [kg] / height^2 [m^2]. Low-density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula. IR was quantified by homeostatic model of assessment-insulin resistance (HOMA-IR) using the formula HOMA-IR = (glucose [mmol/L] * insulin [μU/mL]) / 22.5 [11].

NAFLD patients were classified into those with NAFL or NASH according to the criteria of NAFLD Activity Score (NAS) [12]. Steatosis grade, fibrosis stage, lobular and portal inflammation, and ballooning were categorized based on the classification of NASH Clinical Research Network [12]. Regarding fibrosis stage, cirrhosis (grade 4) was not included, as an exclusion criterion.

2.1. *Statistical Analysis*

Continuous data are presented as mean ± standard error of the mean (SE). Categorical data are presented as numbers. Kolmogorov–Smirnov test was used to check the normality of distributions of continuous variables. Independent samples *t* test or Mann–Whitney test was used for between group comparisons, in cases of two groups of continuous variables. Chi-square test or Fisher's exact test was used to compare categorical variables in four levels, based on: 1) history of *Hp* eradication treatment; 2) anti-*Hp* IgG seropositivity; 3) ^{13}C urea breath test positivity (only for between NAFL and NASH comparisons); 4) combination of history of *Hp* eradication treatment and/or *Hp* seropositivity (and/or ^{13}C urea breath test positivity). Spearman's coefficient (rs) was used for binary correlations. Binary logistic regression analysis was used mainly to identify independent predictors for NASH. For the need of this analysis, variables without normal distribution were logarithmically transformed. Statistical analysis was performed with SPSS 17.0 for Windows (SPSS, Chicago, IL). Significance was set at $P < .05$. *Post-hoc* power analysis was performed by G*Power software (University of Heinrich-Heine, Dusseldorf, Germany).

3. Results

Twenty-eight patients with NAFLD (15 with NAFL and 13 with borderline or definite NASH) and 25 controls were included in this series. Comparative data of study groups are presented in Table 1. There were no statistically significant differences between groups in gender, age, weight, BMI, waist circumference and hsCRP. NAFLD group had statistically higher AST, ALT, GGT, glucose, insulin, HOMA-IR and TNF-α, whereas lower total and HMW adiponectin, compared to control group (Table 1).

Hp-related data in NAFLD and control group are presented in Table 2. Statistically higher rates of anti-*Hp* IgG seropositivity ($P = .038$) were observed in NAFLD group compared to control group. Six NAFLD patients (21.4%) had received eradication treatment in the past (12 months prior to enrollment), whereas only one control; however, the difference was not statistically significant ($P = .104$). Three of NAFLD patients having received eradication treatment in the past were anti-*Hp* IgG seronegative. When information from history (eradication treatment) and anti-*Hp* IgG seropositivity were combined, only two NAFLD patients neither were *Hp* seropositive nor did they have a history of eradication treatment compared to 11 control subjects ($P = .002$). The same analysis, plus data derived from ^{13}C urea breath test, was repeated within the NAFLD group (NAFL vs. NASH patients were compared; Table 3). Notably, none of the NASH patients had received *Hp* eradication treatment in contrast to six NAFL patients. There were no statistically significant differences regarding

Table 1 – Comparative data of study groups.

	Control group (n = 25)	NAFLD group (n = 28)	P value*	Reference values
Women / Men (N)	20 / 5	21 / 7	.664	-
Age (years)	53.8 ± 1.7	55.2 ± 1.7	.570	-
Weight	82.3 ± 2.6	85.9 ± 3.0	.377	
BMI (kg/m^2)	30.8 ± 0.8	32.9 ± 1.0	.062	20–25
Waist circumference (cm)	101 ± 2	106 ± 2	.051	≤80 (females) ≤94 (males)
AST (U/L)	20.0 ± 0.8	32.6 ± 2.3	<.001	10–31
ALT (U/L)	19.6 ± 1.8	45.4 ± 4.0	<.001	10–34
GGT (U/L)	19.6 ± 3.4	49.6 ± 8.4	.002	0–38
Glucose (mg/dL)	89 ± 2	105 ± 5	.006	60–100
Insulin (μU/mL)	5.2 ± 0.8	12.0 ± 1.5	<.001	6–27
HOMA-IR	1.19 ± 0.21	3.25 ± 0.47	<.001	-
hsCRP (mg/L)	3.7 ± 0.4	5.0 ± 0.9	.300	<5
Adiponectin total (μg/mL)	7.8 ± 0.8	5.0 ± 0.5	.006	-
Adiponectin HMW (μg/mL)	3.4 ± 0.3	2.4 ± 0.3	.015	-
TNF-α (pg/mL)	9.9 ± 0.7	17.3 ± 1.7	.002	-

Data are presented as mean ± standard error of the mean (SE) or numbers.
Abbreviations: ALT, alanine transaminase; AST, serum aspartate transaminase; BMI, body mass index; GGT, gamma-glutamyl transferase; HMW, high molecular weight; HOMA-IR, homeostatic model assessment insulin resistance; hsCRP, high sensitivity C-reactive protein; NAFLD, nonalcoholic fatty liver disease; TNF, tumor necrosis factor.
* Between groups comparison (independent *t* test, or Mann–Whitney test or Chi-Square test).

Table 2 – *Hp*-related data in control and NAFLD groups.

	Control group (n = 25)	NAFLD group (n = 28)	P value*
History of *Hp* eradication treatment	1 (4.0)	6 (21.4)	.104
Anti-*Hp* IgG seropositivity	14 (56.0)	23 (82.1)	.038
Combination of history of *Hp* eradication treatment and/or *Hp* seropositivity	14 (56.0)	26 (92.9)	.002

Data are presented as absolute numbers (percentage).
Abbreviations: *Hp*, *Helicobacter pylori*; IgG, immunoglobulin G; NAFLD, nonalcoholic fatty liver disease.
* Chi-Square test or Fischer's exact test.

Table 3 – *Hp*-related data in NAFL and NASH subgroups.

	NAFL subgroup (n=15)	NASH subgroup (n=13)	P value *
History of *Hp* eradication treatment	6 (40.0)	0 (0.0)	.018
Anti-*Hp* IgG seropositivity	12 (80.0)	11 (84.6)	.750
^{13}C urea breath test positivity	4 (26.7)	7 (53.8)	.142
Combination of history of *Hp* eradication treatment and/or *Hp* seropositivity and/or ^{13}C urea breath test positivity	15 (100.0)	11 (84.6)	.206

Data are presented as absolute numbers (percentage).
Abbreviations: *Hp*, *Helicobacter pylori*; IgG, immunoglobulin G; NAFL, simple nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis.
* Chi-Square test or Fischer's exact test.

anti-*Hp* IgG, ^{13}C urea breath test or their combination between NAFL and NASH patients. Similar results to those presented in Table 3 were drawn, when the analysis was repeated by dividing NAFLD group into three sub-groups (NAFLD, borderline NASH and definite NASH).

Subsequently, study parameters were compared between participants without (group 1; n=13) and with (group 2; n=40) history of *Hp* eradication treatment and/or *Hp* seropositivity (Table 4). Group 2 had significantly higher age, AST, ALT, glucose, insulin, HOMA-IR and TNF-α compared to group 1. There were no between group statistically significant differences in weight, BMI, waist circumference, hsCRP, total and HMW adiponectin, and GGT, although BMI, total and HMW adiponectin showed a trend towards higher levels in group 2. Similar data were observed when study parameters were compared between participants without (n=16) and with (n=37) *Hp* seropositivity (data not shown because of their similarity to the above mentioned). Furthermore, anti-*Hp* IgG titer was positively correlated with age (rs=0.312, *P*=.023), total adiponectin (rs=0.415, *P*=.002), HMW adiponectin (rs=0.412, *P*=.002), TNF-α (rs=0.426, *P*=.006). No correlation between anti-*Hp* IgG and the rest of the study parameters was observed.

These analyses were not repeated within NAFLD patients only, because of the small number of patients in either group 1 (n=2) or *Hp* seronegative (n=5), respectively. When the study parameters were compared between NAFLD patients negative (n=17) or positive (n=11) in ^{13}C urea breath test, statistically significant higher levels of both total and HWM adiponectin were observed in ^{13}C urea breath test positive patients (total adiponectin: 3.9±0.5 vs. 5.0±0.8 μg/mL, respectively, *P*=.048; HMW adiponectin: 1.9±0.3 vs. 2.8±0.4 μg/mL, respectively, *P*=.036). None of the rest parameters provided statistically significant difference between the groups. Notably, glucose (*P*=.913), insulin (*P*=.978) and HOMA-IR (*P*=.956) were very similar between these groups (data not shown). Regarding liver histology, there were no statistically significant differences in steatosis grade, fibrosis stage, lobular or portal inflammation, or ballooning, when NAFLD patients (n=28) were divided according to *Hp* seropositivity or ^{13}C urea breath test positivity or combination of history of *Hp* eradication treatment and/or *Hp* seropositivity and/or ^{13}C urea breath test positivity.

Table 4 – Comparative data between participants with and without history of *Hp* eradication treatment and/or anti-*Hp* IgG seropositivity.

	Participants without history of *Hp* eradication treatment or *Hp* seropositivity (group 1; n=13)	Participants with history of *Hp* eradication treatment and/or *Hp* seropositivity (group 2; n=40)	P value *
Age (years)	49.2±2.6	56.3±1.2	.010
Weight	85.8±4.1	83.7±2.3	.656
BMI (kg/m^2)	29.8±0.9	32.4±0.8	.084
Waist circumference (cm)	101±3	104±2	.284
AST (U/L)	20.9±1.6	28.6±1.9	.004
ALT (U/L)	23.6±3.5	36.3±3.5	.015
GGT (U/L)	28.6±6.3	37.7±6.4	.642
Glucose (mg/dL)	87±2	101±4	.003
Insulin (μU/mL)	4.6±0.8	10.0±1.2	.020
HOMA-IR	1.01±0.19	2.64±0.36	.012
hsCRP (mg/L)	4.0±0.5	4.6±0.7	.476
Adiponectin total (μg/mL)	5.3±0.8	6.7±0.6	.244
Adiponectin HMW (μg/mL)	2.4±0.4	3.1±0.3	.172
TNF-α (pg/mL)	9.7±1.7	15.7±1.6	.030

Data are presented as mean±standard error of the mean (SE).
Abbreviations: ALT, alanine transaminase; AST, serum aspartate transaminase; BMI, body mass index; GGT, gamma-glutamyl transferase; HMW, high molecular weight; HOMA-IR, homeostatic model assessment insulin resistance; *Hp*, *Helicobacter pylori*; hsCRP, high sensitivity C-reactive protein; IgG, immunoglobulin G; TNF, tumor necrosis factor.
* Between groups comparison (independent *t* test, or Mann–Whitney test).

Binary logistic regression analysis was first performed within the sum of participants (n=53), with the dependent variable being the diagnosis of NAFLD. Although log(HOMA-IR) could independently predict NAFLD (*P*=.004), *Hp* seropositivity (*P*=.230) could not predict NAFLD independently from age, gender, waist circumference and log(HOMA-IR). This model provided chi-square=21.9 (*P*=.001) and *R* square=0.426. However, when the combination of history of *Hp* eradication treatment and/or *Hp* seropositivity was replaced with single *Hp* seropositivity, both *Hp* infection (*P*=.034) and log(HOMA-IR) (*P*=.007) could independently predict NAFLD. This model provided chi-square=26.0 (*P*<.001) and *R* square=0.489.

Finally, binary logistic regression analysis was performed within NAFLD patients (n=28), with the dependent variable being the histological diagnosis of NAFL or NASH. *Hp* seropositivity (*P*=.951) could not predict NASH independently from age, gender, waist circumference and log(HOMA-IR). This model provided chi-square=3.7 (*P*=.594) and *R* square=0.165. Likewise, ^{13}C urea breath test positivity (*P*=.278) could not independently predict NASH in a similar regression model (chi-square=4.6; *P*=.467; *R* square=0.165). The combination of history of *Hp* eradication treatment and/or *Hp* seropositivity

and/or ^{13}C urea breath test positivity could also not independently predict NASH.

4. Discussion

In this study, *Hp* seropositivity was more frequently observed in biopsy-proven NAFLD patients than in the control group. Both *Hp* infection (assessed by history of *Hp* eradication treatment and/or anti-*Hp* IgG seropositivity) and IR could independently predict NAFLD, when adjusted for each other plus age, gender and waist circumference. Moreover, there were no differences in *Hp* seropositivity or ^{13}C urea breath test positivity between NAFL and NASH subgroup, although more NAFL than NASH patients had a history of *Hp* eradication treatment. Furthermore, neither *Hp* seropositivity nor ^{13}C urea breath test positivity or their combination could independently predict NASH, when adjusted for age, gender, waist circumference and IR.

To our knowledge, this is the first report of positive correlation between anti-Hp IgG and serum total or HMW adiponectin, which is considered to play a pivotal role in the pathogenesis of NAFLD [13,14].

In accordance with the results of a current systematic review showing a trend towards a positive association between *Hp* infection and HOMA-IR [4], in this study, the participants with *Hp* infection (group 2) had higher glucose, insulin and HOMA-IR compared to those without previous or current *Hp* infection (group 1) (Table 4). Interestingly, group **2** had higher ALT, AST and TNF-α than group **1**. Furthermore, the positive correlation between anti-*Hp* IgG titre and total or HMW adiponectin or TNF-α is of note.

Based on these results, it could be hypothesized that *Hp* infection may contribute to the pathogenesis of NAFLD, mainly adding to the first hit of a disease considered to develop through multiple hits [2]; *Hp* infection may trigger TNF-α, whereas adiponectin is secondarily increased to counterbalance the pro-inflammatory cascade. This may be achieved indirectly, through increasing IR, but also directly, given that it could predict NAFLD independently from IR. TNF-α may be a mediator of both direct and indirect effect of *Hp* infection on NAFLD. On the other hand, *Hp* infection does not seem to play a certain role to the progression of NAFL to NASH; both current *Hp* infection or past *Hp* infection did not increase the rates of NASH, and *Hp* infection could not independently predict NASH; however, it is of interest that none of the NASH patients had previously received eradication treatment, but it could be simply attributed to a failure of previous *Hp* infection diagnosis in this subgroup: both the small number of our patients and the fact that *Hp* infection is often asymptomatic may have led to this difference.

To our knowledge, there is possibly one study reporting in Japanese that *Hp* infection was one of the independent risk factors for the development of NAFLD [10]. In another study, the presence of 16 S recombinant RNA of *Hp* spp. on liver samples of a patient with NASH has been reported [8]. This finding was validated in a similar study, in which the 16 S recombinant RNA was found in 5/11 liver samples of NAFLD patients compared with 2/13 controls [9]. However, it remains unclear whether *Hp* in these specimens is an incidental finding or it has the potential to affect the natural course of NAFLD.

The results of this study may be of greater importance in regions with high *Hp* prevalence. More specifically, the prevalence of *Hp* infection in Greece has been reported to be about 50% [15], similar to that found in our control group (Table 2). Despite the lack of population-based studies for the prevalence of NAFLD in Greece, a recent systematic review [16] reported that the prevalence of NAFLD worldwide ranges 6%–35%, with a median of 20%; the prevalence of NAFLD in Italy, which is close to Greece, is approximate to this median (20%). However, based on the high prevalence of obesity in Greece and the results of an autopsy study (showing NAFL 31% and NASH 40%) [17], the prevalence of NAFLD may be higher in general Greek population. The high prevalence of both *Hp* infection and NAFLD renders further research linking them necessary.

This study has certain limitations: 1) The sample size was small; given that this was a pilot study, we did not perform an *a priori* power analysis; however, the "combination of history of *Hp* eradication treatment and/or *Hp* seropositivity" between NAFLD and control group, provided *a post-hoc* power of 99.7%, for type α error 0.05. 2) The controls were not subjected to liver biopsy, due to ethical considerations. 3) Anti-*Hp* IgG cannot distinguish current from old *Hp* infection. 4) ^{13}C urea breath test, which is indicative of current *Hp* infection, was not performed at the control group, due to limited resources. 5) The diagnosis of *Hp* infection was not established by culture or histology representing the practical diagnostic gold standard of *Hp* infection; however, this was beyond the aim of the study, given that *Hp* infection may be only the trigger for NAFLD and, therefore, negative culture or histology does not exclude previous *Hp* infection indicated by *Hp* positive serology. 6) *Hp* was not investigated in liver histological specimens.

In conclusion, *Hp* infection was more frequently observed in NAFLD patients than controls, but there were similar rates between NAFL and NASH patients. This effect may be exerted both directly and indirectly, through increase in IR. *Hp* infection may represent one more hit contributing to the pathogenesis of NAFL, though not to the progression from NAFL to NASH. These results warrant further research with large-scale studies. If confirmed, eradicating *Hp* infection may have certain therapeutic perspectives in NAFLD treatment.

Author contributions

S.A. Polyzos: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; final approval.

J. Kountouras: study concept and design; acquisition of data; interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; final approval; study supervision.

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E. Zafeiriadou: acquisition of data; critical revision of the manuscript for important intellectual content; final approval.

C. Zavos: drafting of the manuscript; critical revision of the manuscript for important intellectual content; final approval.

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Conflict of interest

No potential conflict of interest relevant to this article is declared.

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Association of *Helicobacter pylori* Infection with Colorectal Cancer

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Abstract

Background & Aims: *Helicobacter pylori* (*H. pylori*) and stem cell cross-talk are involved in the tumorigenesis of the upper gastrointestinal tract. The aim of this study was to investigate the possible involvement of *H. pylori* infection in the sequence: chronic inflammation–adenoma–colorectal cancer (CRC) development.

Methods: We investigated the following aspects: a. endoscopic and conventional histological examinations of patients with CRC, colorectal adenomas versus controls to detect the presence of *H. pylori* infection; b. immunohistochemical determination of presence of *H. pylori*; expression of CD44, indicator of cancer stem cells (CSCs) and/or bone marrow-derived stem cells (BMDSCs); expressions of oncogene Ki67 and anti-apoptotic Bcl-2 protein; c. expression of CD45, indicator of immune surveillance locally; and d. correlation of the studied parameters with the presence or absence of *H. pylori*.

Results: In 50 patients with CRC, 25 patients with colorectal adenomas and 10 controls, we found significantly higher presence of *H. pylori* infection in the adenoma (68%) and CRC group (84%) compared with controls (30%). Presence of *H. pylori* infection with accompanying immunohistochemical expression of CD44 in biopsy specimens was found in a high proportion of patients with colorectal adenomas accompanied with moderate/severe dysplasia (88%) and CRC patients with moderate/severe degree of malignancy (91%). Comparable pictures were also obtained for Ki67, Bcl-2 and CD45 immunohistochemical expressions.

Conclusion: *H. pylori* infection by causing a possible chronic inflammatory colon mucosal damage, stimulating CSCs or recruiting BMDSCs and affecting oncogenes and immune surveillance processes may be involved in the sequence: adenoma – dysplasia – CRC development.

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Key words

Helicobacter pylori; colorectal cancer; apoptosis; proliferation; stem cell

Introduction

While the etiology of colorectal cancer (CRC) remains largely unknown,[1] it appears that environmental and hereditary factors play an important role in oncogenesis of the gastrointestinal (GI) tract including the colon. Both of these factors lead either to expression of abnormal oncogenes or to inappropriate expression of normal genes whose derivatives provide the malignant phenotype.[2] The tumor often emerges in foci of chronic inflammatory process-infection of the GI tract; chronic inflammation appears to be the leading cause of several human cancers including CRC[3] and current epidemiological data indicate that over 25% of all cancers are related to chronic infections and other types of unresolved inflammation.[4]

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Regarding *Helicobacter pylori* (*H. pylori*)-associated chronic inflammatory lesion which potentially leads to oncogenesis, apart from upper GI tract, recent evidence suggests that *H. pylori* infection may be involved in colorectal carcinogenesis.[5-7] Recent data also suggest that *H. pylori* is associated with a (small) increase in the risk for both colorectal adenoma and CRC[8] and there is a possible association between hypergastrinemia in CRC patients and *H. pylori* infection, suggesting that the bacterium may be a risk factor for CRC development and via plasma gastrin increase, particularly for left colon cancer.[9,10] It has been shown that gastrin is a specific growth factor for *H. pylori* (causing *H. pylori* growth in a dose-dependent manner),[11] and *H. pylori* infection may be involved in CRC oncogenesis through gastrin and cyclo-oxygenase (COX)-2 overexpression, which promote tumor growth and reduce apoptosis by increasing mRNA expression particularly of anti-apoptotic Bcl-2 protein rather than the apoptotic Bax protein.[12]

Furthermore recent observations point to the possible involvement of *H. pylori* and stem cell cross-talk in GI tumorigenesis. Experimental and preliminary data in human

suggest that *H. pylori* infection is implicated in gastric tumorigenesis through induced chronic gastric inflammation that promotes the recruitment of bone marrow-derived stem cells (BMDSCs) from the circulation into the upper GI tract, which via intestinal metaplasia can contribute to gastric oncogenesis.[13,14]

Theoretically, *H. pylori*-associated localization in the colon causing chronic inflammation may also recruit BMDCs in the intestinal epithelium that may contribute to CRC development and progression, similar to gastric cancer.[1] In this respect, the cancer stem cell (CSC), which derives from transformation of normal stem cell, has been considered to contribute to the onset and progression of cancer including CRC.[15-18] Moreover, the markers of CSCs are similar to those of stem cells of normal tissues, such as the transmembrane adhesion molecule CD44, which is also a marker of stem hematopoietic and tumor colon or gastric cells.[19-25]

It is important to note that *H. pylori* infection may be responsible directly or through inflammation for the increased expression of glycoprotein CD44 observed in gastric cancer[5,26,27] wherein the hematopoietic stem cells appear to participate in its development. Furthermore glycoproteins CD44, which are normally expressed only in the crypts of the intestinal epithelium, are overexpressed in CRC and their overexpression is an early event in the sequence adenoma-CRC development.[28] However, no data exist concerning the likely induced overexpression of these markers in the sequence adenoma-CRC development and progression, including CD44 adhesion molecules in relation to *H. pylori* infection and the concomitant involvement of hematopoietic stem cells and/or CSCs in tumorigenesis of the lower GI tract. Based on the aforementioned data, the aim of this study was: (1) to confirm in a new cohort of patients the initial finding that there is an increased presence of *H. pylori* infection in patients with CRC; (2) to investigate the possible increased prevalence of *H. pylori* infection in preneoplastic conditions of the colon that include colon adenomas; (3) to perform a comparative study on the presence of *H. pylori* infection versus a control group; (4) to test the shaped hypothesis that *H. pylori*-associated localization in the colon is likely to induce recruitment of BMDSCs and/or CSCs activation in the intestinal epithelium which might contribute to the sequence adenoma-CRC development; and (5) to investigate the possible abnormalities of oncogenes and immune surveillance in the reported sequence of colon oncogenesis.

Patients and Methods

From January 2006 to January 2009, among patients who underwent endoscopic control of lower or upper GI tract at the Gastroenterology Outpatient Clinic of the Second Department of Internal Medicine, Aristotle University of Thessaloniki, 50 CRC patients (28 male, mean age 72.54±7.19 years) and 25 patients with colon adenomas (13 male, mean age 61.76±14.08 years) were included. Moreover, as a control group, we included 10 patients with iron deficiency anemia (6 male, mean age 72.8±17.98 years) and absence of macroscopic findings in the endoscopic evaluation of upper and lower GI tract.

Inclusion criteria were: (1) mild iron deficiency anemia; (2) hematochezia, constipation or defecation disorders; (3) absence of macroscopic findings in the endoscopic evaluation of upper GI tract. Exclusion criteria were: (1) coexisting malignancy; (2) inflammatory bowel disease; (3) history of upper GI bleeding; (4) history of operation in the colon; (5) severe cardiovascular disease, respiratory failure, other GI disorders, neuropsychiatric disorders or chronic kidney failure; (7) pregnancy; (8) previous *H. pylori* eradication treatment; (9) reluctance of patients to be included in the research protocol.

All participants gave written consent after they were informed about the research protocol. They study protocol conformed to the principles of the Declaration of Helsinki (1964), and was approved by the Medical Ethics Committee of the hospital (Ippokration University Hospital, Thessaloniki).

Endoscopic evaluation

Patients with CRC were referred for endoscopic evaluation of the lower GI tract in the morning hours (10-11 am) after a 12 h period of fasting and proper bowel preparation receiving 4 L hyperosmolar solution of polyethylene glycol laxative the day before the examination with or without concomitant use of laxative enemas.

Before the endoscopy, sedation with intravenous midazolam 2-5 mg was administered (Dormicum® 15 mg/3 mL, Roche, Athens, Greece). Thereafter, thorough endoscopic evaluation of the colon up until the cecum was performed by two endoscopists (NK, JK) using an endoscope Olympus GIF type V2 (Olympus Optical, Tokyo, Japan); multiple biopsy specimens were obtained with special forceps (Multibyte biopsy forceps, Boston Scientific, Microvasive, Watertown, MA, USA) from the area of the lesion (6-8 specimens), according to the protocol. Presence of *H. pylori* infection was detected with conventional and immunohistochemical staining, and immunohistochemical staining was also used for the expression of transmembrane adhesion molecule CD44 (marker of hematopoietic stem cells and CSCs), oncogene Ki67 (cellular proliferation marker), anti-apoptotic protein Bcl-2 and cell markers of immune surveillance by identifying T lymphocytes (TLs) and B lymphocytes (BLs) (CD45). To achieve this, care was taken to avoid transfection of the biopsy specimens, and the endoscope was disinfected after each endoscopy according to the standard guidelines in an automated washing machine (David 3G plus, Washer disinfector, Athens, Greece).

In the group of patients with adenomas the same protocol for the endoscopic and histological evaluation was applied, while endoscopic polypectomy of adenomas using a snare was also performed at the same time.

In the control group the same protocol was applied for the endoscopic evaluation of the lower GI tract, with additional endoscopic evaluation of the upper GI tract with the endoscope Olympus GIF type V2 (Olympus Optical, Tokyo, Japan) after oropharyngeal anesthesia administration of spray lidocaine 2% (Xylocaine®, AstraZeneca, Athens, Greece) and/or intravenous administration of 10 mg hyoscin (Buscopan® 20 mg/1 mL, Boehringer-Ingelheim, Athens, Greece) to reduce peristalsis and salivation; 3-4 biopsy specimens were obtained from the gastric antrum and corpus to detect *H. pylori* infection with urease test and conventional histological examination. Histological

control of colon biopsy specimens included conventional and immunohistochemical determination of the presence of *H. pylori* infection and additional immunohistochemical detection of the expression of transmembrane adhesion molecule CD44.

Histological evaluation of biopsy specimens

Urease test in gastric mucosa

In one biopsy specimen from the antrum and one from the corpus of the stomach *H. pylori* infection was assessed using urease test as described previously.[29]

Conventional evaluation of gastric mucosa biopsy specimens

The gastric biopsy specimens were stained with Crezyl fast violet and/or hematoxylin-eosin for histological presence of *H. pylori* infection. Furthermore, the same staining was used for evaluation of possible presence of gastritis.

In the present study as the practical diagnostic "gold standard" for diagnosis of *H. pylori* infection was considered the finding of microorganisms in the histological examination of gastric mucosa biopsy specimens,[30,31] while as adjunct diagnostic test, the positive urease test in gastric mucosa biopsy specimens was considered.

Gastritis was classified according to Sydney classification.[32,33]

Conventional histological evaluation of colon mucosa biopsy specimens

Colon biopsy specimens were stained with the same Crezyl fast violet and/or hematoxylin-eosin stains for histological presence of *H. pylori* infection and diagnosis of tumors.

Histological assessment of colon mucosa biopsy specimens

Apart from the histological examination of the upper GI tract that involved only the control group, in all colon mucosa biopsy specimens conventional (presence of *H. pylori* infection, tumor type and differentiation) and immunohistochemical (presence of *H. pylori*, expression of CD44, markers of TLs, BLs (CD45) and oncogenes Ki67 and Bcl-2) evaluations were made by two expert pathologists (I.V., C.N.) who were not aware of the history, clinical presentation of patients and endoscopic findings.

Specifically, the conventional evaluation included the histological type of tumor, differentiation, infiltration of lymphatic, vascular or perineural spaces and the presence of *H. pylori* infection in the neoplastic tissue.

Immunohistochemical evaluation

Immunohistochemical analysis for expression of CD44 and CD45 in cancer and adenomatous colon tissue was made using mouse monoclonal antibodies (DakoCytomation Denmark and Novocastra, respectively), while for the immunohistochemical analysis of the presence of *H. pylori* we used the polyclonal mouse antibody anti-*H. pylori* antibody (DAKO):

- CD44 (DakoCytomation, Denmark). Murine monoclonal antibody provided in liquid form, and used at a dilution 1:50; indicates the presence of hematopoietic stem cells and/or CSCs.

- CD45RO (Novocastra). Murine monoclonal antibody provided in liquid form, and used at a 1:100 dilution; marks mainly lymphocytes (T and B), and moreover monocytes and eosinophils. It does not recognize normal erythrocytes and platelets.

- Anti-*H. pylori* antibody (DAKO). Polyclonal murine antibody provided in liquid form and used at a dilution of 1:50; indicates the presence of *H. pylori* bacteria.

Colon mucosa biopsy specimens were also evaluated immunohistochemically for Ki67 and Bcl-2 expressions (Biogenex Laboratories, Inc., San Ramon, CA, USA).

Grading of immunohistochemical staining of Ki67 and Bcl-2

The expression of Ki67 and Bcl-2 were estimated and scored quantitatively by the proportion of cells stained.

Statistical analysis

Data are presented as mean ± standard deviation (SD) for continuous variables and numbers and/or percentages for categorical variables. The normality of distribution was tested by the Kolmogorov-Smirnov test. Independent t-test or Mann-Whitney test were used to test for differences between two groups of continuous variables. One-way ANOVA or Kruskal-Wallis tests, followed by Bonferroni post-hoc analysis, were used to test for differences among more than two groups of continuous variables. Chi-square or Fischer's exact test were used for between group differences in categorical variables. Statistical analysis was performed using SPSS 17.0 statistical software (SPSS Inc, Chicago, IL). All p-values reported were bidirectional (2-tailed). Statistical significance was set at $p<0.05$.

Results

Mean age was significantly higher in CRC and control groups compared to the adenoma group (71.3±9.7, 72.8±18 vs. 64±14 years, respectively, $p=0.049$), whereas no difference was observed between CRC and control groups (71.3±9.7 vs. 72.8±18 years, respectively, $p=0.808$). Among the 3 study groups, no difference was observed in the sex ratio ($p=0.816$) and in socioeconomic status.

In the total CRC group, endoscopic presence and histologically confirmed malignancy was found in a proportion of patients: 1) 32% (16/50) in the rectum; 2) 30% (15/50) in the sigmoid and descending colon; 3) 4% (2/50) in the transverse colon; and 4) 34% (17/50) in the ascending colon and cecum (**Table 1**). Based on the location of the CRC in the left colon (rectum, sigmoid, descending and distal transverse colon) and the right colon (proximal transverse, ascending and cecum), the analysis showed increased localization of malignancy in the left colon [in 62% (31/50) of patients]. Regarding the characteristics of histological severity, low-grade malignancy was shown in 9 CRC patients, moderate in 37 and high in 4.

As shown in **table 1**, histological presence of *H. pylori* infection, confirmed by immunohistochemical staining, was observed in 84% (42/50) of patients with CRC and 68% (17/25) of patients with adenomas. Compared with the adenoma group, an increased histological presence of *H. pylori* was shown in the CRC group, almost reaching a statistical significance ($p=0.051$). Histological presence of *H. pylori* infection confirmed by

Table 1. Location of histological lesions of patients with CRC and adenomas in the total of patients and in the subgroups of *Hp* (+) patients

Location	Total CRC patients (%) *	*Hp* (+) CRC patients (%) #	Total adenomas patients (%) *	*Hp* (+) adenomas patients (%)
Rectum	16 (32%)	15 (94%)	7 (32%)	3 (43%)
Sigmoid and descending colon	15 (30%)	12 (80%)	11 (50%)	8 (82%)
Transverse colon	2 (4%)	1 (50%)	2 (9%)	1 (50%)
Ascending colon and cecum	17 (34%)	14 (82%)	2 (9%)	2 (100%)
Total	50 (100)	42 (84%)	22 (100)†	14 (64%)

* Percentages present proportions of patients to the sum of patients.
# Percentages present proportions of *Hp* (+) patients to the patients of each subgroup.
† 3 patients had adenomas in two different locations and were not included in this table.
CRC, colorectal cancer; *Hp*, *Helicobacter pylori*.

Table 2. Immunohistochemical expression of CD44, CD45, Ki67 and Bcl-2 in patients with CRC and adenomas in the total of patients and in the subgroups of *Hp* (+) patients

Location	Total CRC patients (%) *	*Hp* (+) CRC patients (%) #	Total adenomas patients (%) *	*Hp* (+) adenomas patients (%) #	Controls	*Hp* (+) controls
CD44 (+)	45 (90)	38 (91%)	21 (84%)	13 (81%)	1 (10)	1 (33)
CD45 (+)	40 (80)	34 (81%)	2 (8%)	1 (6%)	NA	NA
Ki67 (+) †	17 (100)	13 (100%)	13 (87%)	8 (80%)	NA	NA
Bcl-2 (+) †	16 (53)	13 (50%)	6 (40%)	4 (40%)	NA	NA

NA, not available.
*: Percentages present proportions of patients to the sum of patients.
#: Percentages present proportions of positive patients to the sum of *Hp* (+) patients.
†: Measured in a subgroup of patients.
CRC, colorectal cancer; *Hp*, *Helicobacter pylori*.

immunohistochemical method was observed in normal colon mucosa in 30% (3/10) of controls. Furthermore, histological presence of *H. pylori* infection and positive urease test on gastric mucosa was observed in 50% (5/10) of controls.

In the CRC group, statistical analysis showed no significant increase in the presence of *H. pylori* infection in patients with CRC in the left colon versus right colon (n=27 vs. 15 patients, respectively, p=0.459). A similar nonsignificant increase in *H. pylori* infection was seen also in patients with adenomas in the left compared to the right colon (n=11 vs. 3 patients, respectively, p=0.601). In 2 patients with polyps in both sides of the colon (ascending-descending and cecum-rectum), presence of *H. pylori* infection was also observed.

Regarding the features of histological severity in CRC group, presence of *H. pylori* infection was observed in 89% (8/9) of patients with mild and 83% (34/41) of patients with moderate/severe grade. Likewise, presence of *H. pylori* infection in the adenoma group was observed in 50% (6/12) of patients with mild and 80% (8/10) of patients with moderate/severe dysplasia.

Immunohistochemical expression of CD44 was found in 90% (45/50) of patients with CRC, 84% (21/25) of patients with adenomas and only in 10% (1/10) in the normal colonic mucosa of controls (p<0.001, **Table 1**). Compared with the adenoma group, a statistically significant increase in expression of CD44 was also observed in the CRC group (p<0.001).

Immunohistochemical CD44 expression was observed in the CRC group in: 1) rectum in 14 patients; 2) sigmoid and descending in 12; 3) transverse in 2; and 4) ascending and cecum in 17. Likewise, immunohistochemical CD44 expression was observed in the adenoma group in: 1) rectum in 6 adenomas; 2) sigmoid and descending in 10; 3) transverse in 2; and 4) ascending and cecum in 2. In 1 patient with polyps in both ascending and sigmoid, positive CD44 expression was also observed.

Immunohistochemical expression of CD44 was found in the proportion of: a) 91% (38/42) *H. pylori*-positive patients with CRC; b) 81% (13/16) *H. pylori*-positive patients with adenomas; and c) 33% (1/3) *H. pylori*-positive control in normal colon mucosa (**Table 2**). Compared with the control group, a statistically significant increase in CD44 expression was observed in *H. pylori*-positive patients with CRC (p=0.014). Compared with the adenoma group, a statistically significant increase in CD44 expression in *H. pylori*-positive patients with CRC was also observed (p=0.002).

In the CRC group with concomitant *H. pylori* infection, statistical analysis showed no significant increase in CD44 expression in CRC patients in the left versus right colon (n=23 vs. 15, respectively, p=0.172). Similar comparison also showed nonsignificant increase in CD44 expression in patients with adenomas in the left versus right colon (n=10 vs. 3, respectively, p=0.727). The 3 patients with double localization of adenomas although did not have simultaneous presence of CD44 and *H. pylori* infection, 2 had only presence of *H. pylori* infection and the third had only CD44.

With respect to the features of histological severity in the CRC group with concomitant *H. pylori* infection, immunohistochemical CD44 expression was observed in 88% (7/8) of patients with mild and 91% (31/34) of patients with moderate/severe grade of malignancy. Similar immunohistochemical CD44 expression in

the adenoma group was observed in 100% (6/6) of patients with mild and 88% (7/8) of patients with moderate/severe dysplasia.

Immunohistochemical CD45 expression was found in 80% (40/50) of CRC patients and 8% (2/25) of adenoma patients. Compared with the adenoma group, a statistically significant increase in CD45 expression was observed in CRC group ($p<0.001$, **Table 3**).

Immunohistochemical CD45 expression was found in the proportion of: a) 81% (34/42) *H. pylori*-positive patients with CRC; and b) 6% (1/16) *H. pylori*-positive patients with adenomas. Compared with the adenoma group, a statistically significant increase in CD45 expression was observed in *H. pylori*-positive patients with CRC ($p<0.001$).

In the CRC group with concomitant *H. pylori* infection, the statistical analysis showed nonsignificant increase in CD45 expression in CRC patients in the left versus right colon (n=21 vs. 13, respectively, $p=0.709$). A similar comparative finding was observed in adenoma patients in left versus right colon (n=1 vs. 0, $p=0.588$).

Regarding the features of histological severity in the CRC group with concomitant *H. pylori* infection, immunohistochemical CD45 expression was observed in 75% (6/8) of patients with mild and 82% (28/34) of patients with moderate/severe grade of malignancy. A comparable immunohistochemical CD45 expression in the group of adenomas with concomitant *H. pylori* infection was observed in 13% (1/8) of patients with moderate/severe dysplasia.

Immunohistochemical Ki67 expression was found in 100% (17/17) of patients with CRC and 87% (13/15) of patients with adenomas (**Table 2**). Compared with the adenoma group, a statistically significant increase in Ki67 expression was observed in CRC group ($p=0.005$).

Immunohistochemical Ki67 expression found in a proportion of: a) 100% (13/13) *H. pylori*-positive CRC patients; and b) 80% (8/10) *H. pylori*-positive adenoma patients (**Table 2**). Compared with the group of adenomas, a statistically significant increase in Ki67 expression was observed in *H. pylori*-positive CRC patients ($p=0.01$).

In the CRC group with concomitant *H. pylori* infection, the statistical analysis showed no significant difference in Ki67 expression in CRC patients in the left versus right colon (n=7 vs. 6, respectively, $p=0.592$). A similar comparison showed no significant increase in Ki67 expression in adenoma patients in left versus right colon (n=6 vs. 1, respectively, $p=0.284$).

Regarding the features of histological severity in the CRC group with concomitant *H. pylori* infection, immunohistochemical Ki67 expression was observed in 100% (4/4) of patients with mild and 100% (9/9) of patients with moderate/severe grade of malignancy. A comparable immunohistochemical Ki67 expression in the adenoma group with concomitant *H. pylori* infection was observed in 80% (4/5) of patients with mild and 75% (13/4) of patients with moderate/severe dysplasia.

Immunohistochemical Bcl-2 expression was found in 53% (16/30) of patients with CRC and 40% (6/15) of patients with adenomas. Compared with the adenoma group, a nonsignificant increase in Bcl-2 expression was observed in the CRC group ($p=0.618$, **Table 2**).

Immunohistochemical Bcl-2 expression was found in the proportion of: a) 50% (13/26) *H. pylori*-positive patients with CRC; and b) 40% (4/10) *H. pylori*-positive patients with adenomas. Compared with the adenoma group, no statistically significant increase in Bcl-2 expression was observed in the group of *H. pylori*-positive CRC patients ($p=0.802$).

In the CRC group with concomitant *H. pylori* infection, the statistical analysis showed no significant difference in Bcl-2 expression in patients with CRC in the left versus right colon ($p=0.235$). A similar finding was observed in adenoma patients in left versus right colon ($p=0.477$).

Regarding the features of histological severity in the CRC group with concomitant *H. pylori* infection, immunohistochemical Bcl-2 expression was observed in 83% (5/6) of patients with mild and 40% (8/20) of patients with moderate/severe grade of malignancy. Likewise, immunohistochemical Bcl-2 expression in the adenoma group with concomitant *H. pylori* infection was observed in 67% (4/6) of patients with mild dysplasia.

Discussion

This series shows for the first time, the comparative presence of *H. pylori* bacteria in colon biopsy samples in a small proportion of control subjects (30%) and progressively in a significant proportion of patients with colon adenomas (68%) and mainly CRC (84%). In particular, comparative presence of *H. pylori* bacteria in biopsy samples of colon adenomas was observed in 50% of patients with mild dysplasia and in a higher percentage (80%) with moderate/severe dysplasia. In the CRC group, presence of *H. pylori* bacteria was observed in a large proportion of patients with mild and moderate/high-grade malignancy (89% and 83%, respectively). Of note, the histological presence of *H. pylori* bacteria was detected by conventional staining (Crezyl fast violet and/or hematoxylin-eosin) and was confirmed by immunohistochemical testing. These findings, referring to a small number of patients, may suggest the potential involvement of *H. pylori* infection in the sequence: transition of colon epithelium to adenoma – mild/moderate/severe dysplasia – development/progression of CRC.

To our knowledge, there are no comparative data in the international literature; the existing data refer only to patients with adenomas and/or CRC, they are controversial and have not cast light on the degree of involvement of *H. pylori* in the reported sequence: colon epithelium – adenoma – dysplasia – CRC. However, the data in the literature show, in their majority, a small but statistically significant increase in the incidence of *H. pylori* infection in adenomas and CRC.[34,35]

In particular, relevant epidemiological studies, using a variety of diagnostic tests to detect *H. pylori* infection (serological detection of anti-*H. pylori* IgG antibodies, PCR, histology, urea breath test and serum gastrin levels), that investigated the possible association of *H. pylori* infection with adenomas and/or CRC seem to be contradictory.[6,36-45] Specifically, by evaluating 16 epidemiological studies that investigated the relationship between adenomas or CRC and the prevalence of *H. pylori* infection, only six of them showed a statistically significant association between *H. pylori* seropositivity and CRC (odds ratio, 1.4-4.0) while the

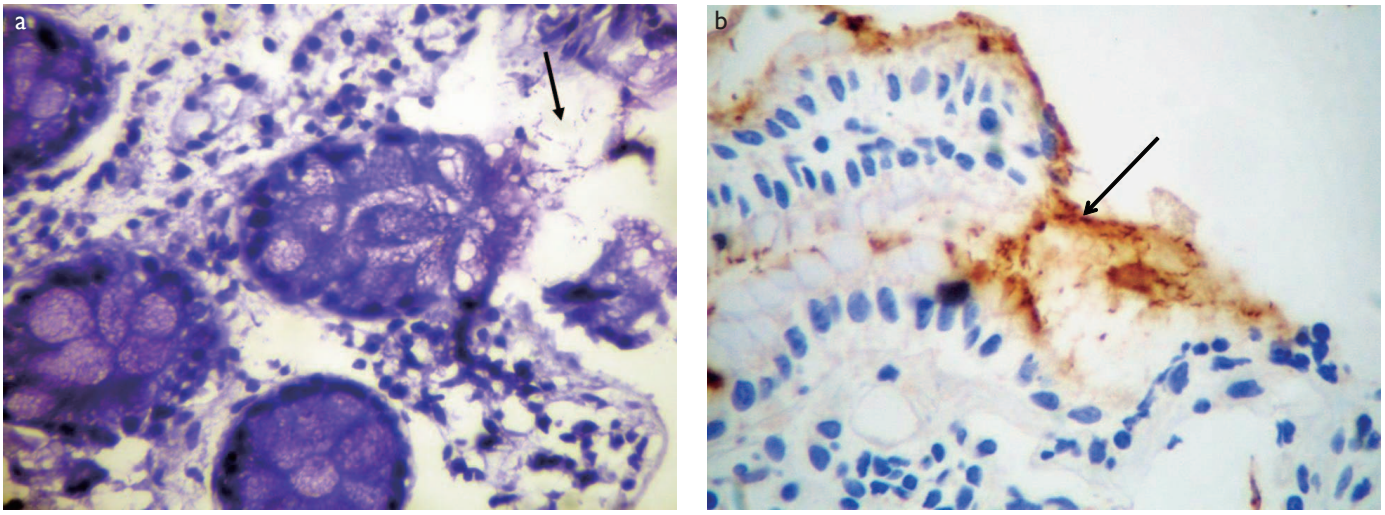

Figure 1. a, Histological presence of *H. pylori* bacteria on the surface of colon adenoma biopsy specimen (Crezyl fast violet staining); b, Histologic confirmation of *H. pylori* bacteria on the surface of biopsy sample of colon adenoma (immunohistochemical staining with anti-*H. pylori* antibody (DACO)]. *H. pylori*, *Helicobacter pylori*..

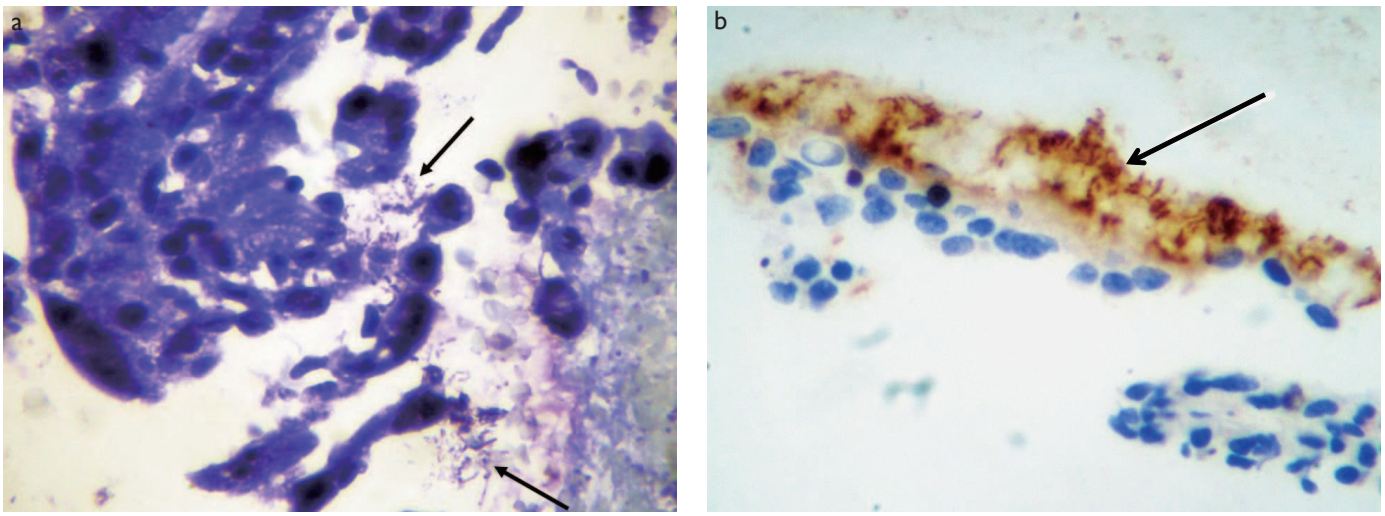

Figure 2. a, Histological presence of *H. pylori* bacteria on the surface of colon cancer biopsy specimen (Crezyl fast violet staining); b, Histologic confirmation of *H. pylori* bacteria on the surface of biopsy sample of colon cancer (immunohistochemical staining with anti-*H. pylori* antibody (DACO)]. *H. pylori*, *Helicobacter pylori*.

others showed no statistically significant association between *H. pylori* seropositivity and colorectal neoplasia.[1] The conflicting findings of the reported studies may be attributed, at least in part, to differences in selection of the controls, to a heterogeneous patient population, to differences in the methodology, to inadequate consideration of possible variables, and to the limited power to detect associations due to the small sample sizes in most studies. It should be particularly underscored that the serological presence of *H. pylori* infection does not differentiate the current from past *H. pylori* infection. Only the presence of active *H. pylori* infection triggers and perpetuates the inflammatory immune reactions by induction and expression of various inflammatory mediators that can lead to gastrointestinal tumorigenesis.

It is important to note that our study was based on histological presence of *H. pylori* infection in biopsy samples of normal and neoplastic colon tissue to all participants and confirmed by immunohistochemical method of the *H. pylori* presence in adenomatous tissue (**Fig. 1a** and **1b**) and CRC (**Fig. 2a** and **2b**).

Although culture is the theoretical gold standard for the detection of the bacterium, it has been shown that there is an excellent correlation with the histological method detecting *H. pylori* infection.[31] Furthermore, because the culture technique is difficult requiring specialized laboratory, for most studies the histological diagnostic method is the practical gold standard detecting active *H. pylori* infection,[30,46,47] while the non-invasive urease test is less sensitive, particularly in the elderly.[48]

In this study we observed, compared to the right colon, an increased presence of *H. pylori* in the tumors of the left colon

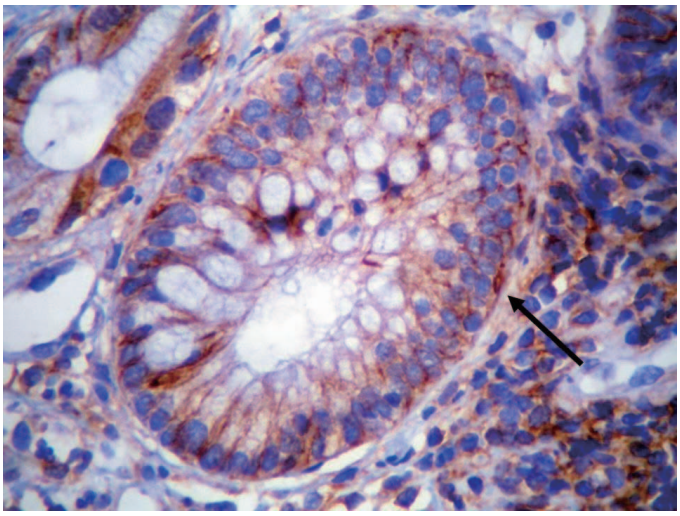

Figure 3. Immunohistochemical staining positive for CD44 in a colon adenoma biopsy specimen from a patient with concomitant *Helicobacter pylori* infection (DakoCytomation, Denmark)

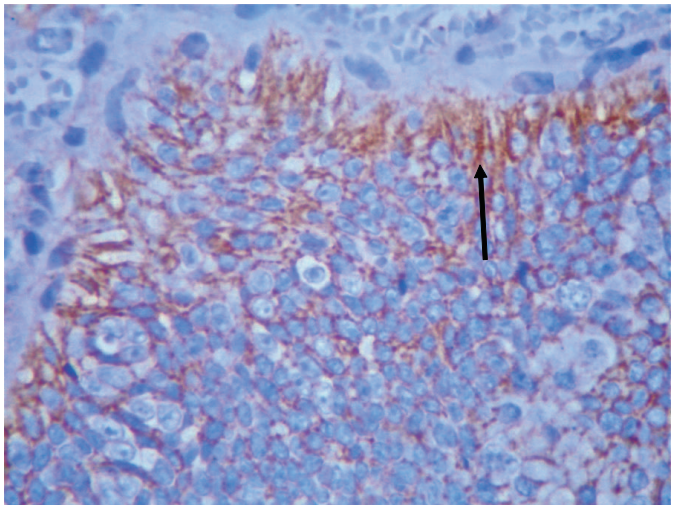

Figure 4. Immunohistochemical staining positive for CD44 in a colon cancer biopsy specimen from a patient with concomitant *Helicobacter pylori* infection (DakoCytomation, Denmark)

(64%),[49] a finding similar to that in a recent large population study showing serological correlation of *H. pylori* infection with the risk of development cancer in the left colon, especially in CagA(+) patients and in the early disease stages.[34] As a possible explanation, it has been postulated that the relationship between *H. pylori* infection and the risk of left colon CRC may be due to increased and prolonged action of gastrin produced from *H. pylori*-infected patients. This view is reinforced by: a) experimental studies suggesting that the mitogenic action of gastrin is mainly confined to the left colon; b) case-control studies showing an increased risk of hypergastrinemia and distal colon adenomas; and c) prospective studies showing gastrin overexpression primarily in patients with left colon CRC compared to another tumor location.[9,34] Furthermore, the findings of this study showed that, compared with the right colon, an increased presence of *H. pylori* infection was observed in the adenomas of the left colon (79%). It was also observed that *H. pylori* infection was more prevalent in tubular and tubulovillous compared to villous adenomas. However, the main limitation of the present series is its small sample size of the patients (mainly of controls) including the number of the last adenomas, limiting the power of the statistical analysis. In contrast, other studies showed increased prevalence of *H. pylori* in villous adenomas compared to other histologic types.[50] Therefore, relative studies are needed in large numbers of patients to draw safe conclusions about the prevalence of *H. pylori* in different histological type adenomas.

Of note, the activation of gastrin via β-catenin is an early event in colon tumorigenesis and may contribute to cancer progression.[51] Specifically, gastrin has been reported to: a) increase the expression of cyclin D1 (via activation of β-catenin/Tcf-4 route) and nuclear antigen PCNA (proliferating cell nuclear antigen) promoting colon carcinogenesis; b) promote the expression of COX-2 in tumor cells of human CRC, suggesting an additional effect of the hormone in the development and progression of the tumor through the induction of COX-2;[52-54] c) have a positive effect on the proliferation of epithelial cells of the intestine, via hypersecretion of inflammatory cytokines [interleukin (IL)-1, IL-8, tumor necrosis factor-α] and growth factors (transforming growth factor-α, epidermal growth factor); d) bind to its cholecystokinin-2 receptors and to activate the ras pathways[54] and as an oncogenic growth factor, it appears to play a role in carcinogenesis of the upper GI tract and the colon;[52,55] and e) increase the expression of vascular endothelial growth factor-A associated with poor prognosis of CRC.[56,57] Moreover, *H. pylori* infection per se is involved in the oncogenesis of colon through overexpression of gastrin and COX-2, which promote tumor growth and decrease apoptosis by increasing mRNA expression particularly of the anti-apoptotic Bcl-2 protein rather than of the apoptotic Bax protein.[58] In this regard, COX-2 induces: prostaglandin E2 synthesis, an increase in cell proliferation, development of mutations in the colon epithelial cells, inhibition of apoptosis of these cells and angiogenesis.

Because *H. pylori* presence in the colon also causes chronic inflammation, this parameter in combination with the aforementioned relative findings of the present series may indicate that, as in the upper GI tract, it seems that there is a similar oncogenetic sequence in the colon comprising: *H. pylori* presence in normal mucosa – induction of *H. pylori*-associated chronic inflammatory lesion – colonic epithelial transformation to adenoma – mild/moderate/severe dysplasia and CRC development/progression. Indeed, relative studies indicate that the passage of *H. pylori* across the intestinal lumen, under appropriate conditions, can cause chronic inflammatory changes in the intestinal mucosa[59] promoting colon tumorigenesis. However, further studies in large numbers of patients are required to thoroughly elucidate the aforementioned sequence. The possible confirmation of this sequence might have practical clinical interest because the eradication of *H. pylori* infection can halt the progression of this sequence particularly in its early stages that, as with the upper GI tract, they are likely to be reversible.

An additional finding of this study showed comparative immunohistochemical expression of CD44 in a small proportion

of controls (10%) and progressively in a significant proportion of tumor tissue of patients with colon adenomas (84%) and CRC (90%). In particular, comparative presence of *H. pylori* bacteria with concomitant immunohistochemical expression of CD44 in biopsy specimens of colon adenomas was observed in all patients with mild dysplasia and in 88% of patients with moderate/severe dysplasia (**Fig. 3**). In the CRC group, it was also observed presence of *H. pylori* bacteria with concomitant immunohistochemical expression of CD44 in a large proportion of patients with mild and moderate/high-grade malignancy (88% and 91%, respectively)(**Fig. 4**). These findings, involving limited numbers of patients, may suggest the involvement of *H. pylori* infection via induction of CD44 expression in the sequence: normal colonic epithelium transition to adenoma – dysplasia – CRC development.

There are no relative comparative data in the literature reporting any association between *H. pylori* infection and CD44 in the context of the aforementioned sequence.

CD44 appears to be an important indicator of CSC involved in tumorigenesis of the GI system including the colon; the CSC, as already mentioned, presents features of normal mature stem cells such as self-renewal, multipotency and differentiation responsible for the initiation and progression of cancer.[60] Moreover, CD44 is also a marker of BMDSCs.[61]

Based on the aforementioned CD44 data, the findings of this study might indicate that, apart from the mechanism of *H. pylori*-associated localization in the colon that causes, via induction of inflammation, the mentioned carcinogenic sequence, there may exist two additional mechanisms for the *H. pylori*-CD44(+)-associated colon oncogenesis: a) through induction of CD44(+) CSC of the colon, and/or b) through recruitment of BMDSCs from the circulation to the colonic mucosa, which, in their attempt to repair the *H. pylori*-associated chronic inflammatory damage of the colonic mucosa might acquire a malignant phenotype thereby inducing tumorigenesis. Relevant studies in the upper GI tract show that *H. pylori*, either directly or through the local inflammatory response, is responsible for the increased expression of CD44 and the isoform of CD44 v9, suggesting a possible *H. pylori*-mediated induction of CD44(+) gastric CSCs involved in the development and progression of gastric cancer.[18] Correspondingly, this study suggests that *H. pylori* may cause similar induction of CD44(+) CSCs in the colon, involved in sequence: *H. pylori* CD44(+)-associated chronic inflammatory lesion – colonic epithelial transformation to adenoma – CRC development. However, this potential consideration requires thorough future elucidation and a large number of patients.

Furthermore, studies in animals and humans indicate that the *H. pylori*-associated chronic inflammatory gastric mucosal damage may lead to loss of the gastric stem cell located in the "niche" of the gastric crypts and failure to repair the damage. Subsequently, BMDSCs are recruited from the circulation to the gastric mucosa, which, in an attempt to repair the gastric damage, exhibit an ability to induce gastric tumorigenesis.[13,18] According to the findings of this study, it is also speculated that *H. pylori* CD44(+)-associated chronic inflammatory damage of the colonic mucosa results in loss of colon stem cell and failure to repair the damage. Subsequently, CD44(+)-BMDSCs are probably recruited from the circulation into the colonic mucosa, which, in their attempt to repair the epithelium, acquire a malignant phenotype, thereby involving in *H. pylori* CD44(+)-related oncogenic sequence of the colon.[49] Similar data are likely to apply to *H. pylori* CD44(+)-related chronic inflammatory lesion involved in colon tumorigenesis of patients with inflammatory bowel disease.[62] However, again, future relevant studies in large numbers of patients are required to unravel in depth the mechanisms of the reported sequences.

Regarding Ki67, the findings of this study showed comparative presence of *H. pylori* bacteria with concomitant Ki67 immunohistochemical expression in biopsy samples of colon adenomas in 80% of patients with mild and 75% with moderate/severe dysplasia. In the CRC group, comparative presence of *H. pylori* bacteria with concomitant Ki67 immunohistochemical expression was observed in all patients with mild and moderate/severe malignancy. These findings involving limited numbers of patients, may suggest the involvement of *H. pylori* infection, via induced and progressively increased expression of the cell proliferation marker (Ki67), in the sequence: adenoma – CRC development and progression. No similar comparative data exist in the literature that correlate *H. pylori* infection with Ki67 with respect to the reported sequence.

Concerning the colon, relative studies show increased Ki67 expression in all adenomas, particularly rectal, and in adenomas with severe dysplasia.[63-65] Therefore, determining Ki67 has a prognostic significance in tumor progression.[65,66]

Based on the reported data on Ki67, the findings of this study further suggest the following additional mechanism of *H. pylori* Ki67(+)-associated cancinogenic sequence: gradual increase in Ki67 proliferation index from the normal colon epithelium to adenoma, dysplasia, CRC development and progression, suggesting that eradication of the bacterium may halt this sequence. However, future studies in large numbers of patients are required to elucidate this mechanism of *H. pylori* infection involvement in CRC development and progression.

Regarding the protein Bcl-2, this study shows for the first time, the comparative presence of *H. pylori* bacteria with concomitant immunohistochemical Bcl-2 expression in colon biopsy samples progressively to 40% of the tumor tissue of patients with colorectal adenomas and 50% of all patients with CRC. In particular, comparative presence of *H. pylori* bacteria with concomitant immunohistochemical Bcl-2 expression in biopsy samples of colon adenomas was observed in 67% of patients with mild dysplasia. In the CRC group comparative features were observed in 83% of patients with mild degree of malignancy and in a lower proportion (40%) with moderate/severe degree of malignancy. These findings, pertaining to small numbers of patients, may also suggest the involvement of *H. pylori* infection via induction of Bcl-2 anti-apoptotic protein in the sequence: adenoma – CRC development. No similar comparative data in the literature have been published correlating *H. pylori* infection with Bcl-2 in the context of the reported sequence. Based on the aforementioned data on Bcl-2, the findings of this study show that, in line with the upper GI tract, the following additional mechanism of *H. pylori* Bcl-2(+)-associated sequence in the colon is also likely to apply: a predominant anti-apoptotic activity of Bcl-2 versus the

apoptotic action of Bax in normal colonic epithelium – adenoma development – dysplasia – CRC, suggesting that eradication of the bacterium may also halt this sequence. In this respect, therapeutic strategies are aimed at Bcl-2 [using endogenous small RNAs (microRNAs) that negatively regulate gene expression at a transcriptional level] to address CRC.[67] However, future relative studies in large numbers of patients are required to elucidate the mechanism of *H. pylori* infection involvement in the development and/or progression of CRC.

Finally, concerning CD45 (assessing mainly T and B lymphocytes), as a marker of possible immune surveillance of the host, this study showed for the first time, the comparative presence of *H. pylori* bacteria with concomitant CD45 immunohistochemical expression in colon biopsy specimens gradually in a small proportion of tumor tissue of patients with colon adenomas (6%) and in a significant proportion of patients with CRC (81%). Specifically, comparative presence of *H. pylori* bacteria with concomitant CD45 immunohistochemical expression in biopsy samples of colorectal adenomas was observed only in moderate/severe dysplasia in 13% of patients. In the CRC group, comparative features were observed in 75% of patients with mild and in 82% with moderate/severe degree of malignancy. These findings pertaining to a limited number of our patients might suggest the progressive increased presence of a degree of immune response of the host in the *H. pylori*-associated sequence: adenoma – CRC development-progression. No relative comparable data in the literature have been published regarding the host immune response against *H. pylori*-associated sequence of colon oncogenesis.

Based on the reported data on CD45, the findings of this study further suggest that there may exist a degree of immune response of the host exerting a protective role against the aforementioned *H. pylori*-associated oncogenic sequence. On the other hand, it is speculated that the escape of *H. pylori* CD45(+) infection from immune surveillance may induce the establishment of the malignant phenotype and the progress of the *H. pylori*-associated sequence: adenoma – dysplasia – CRC development. Moreover, a possible immune escape of the already developed CRC may be associated with tumor spreading and formation of metastases. However, again additional studies in a large number of patients are required to illustrate in depth this field of possible protective or unfavorable operation of the immune surveillance against the *H. pylori*-associated colon oncogenesis.

In conclusion, the findings of the present series show that the presence of *H. pylori* infection in the colon, causing a possible chronic inflammatory mucosal damage, stimulating CSCs or recruiting BMDSCs and affecting oncogenes and immune surveillance processes, may be involved in the sequence: transformation of normal colon epithelium to adenoma – moderate-severe dysplasia – CRC development/progression; the eradication of the bacterium may have a inhibiting effect on colon tumorigenesis. However, prospective studies are required in large numbers of patients to confirm the findings of this study.

Conflicts of interest

The authors declared no conflicts of interest.

Summary Box

What is already known:

- *Helicobacter pylori* (*H. pylori*) and stem cell cross-talk are involved in the tumorigenesis of the upper gastrointestinal tract.
- Recent studies show that a progressive development of genetic alterations in oncogenes and tumor suppressor genes in combination with environmental factors seem to correspond with a progressive clinical and histological sequence of events involving transformation of the normal colonic epithelium to adenoma, dysplasia and finally colorectal cancer (CRC).

What the new findings are:

- The findings of this study show that the presence of *H. pylori* infection in the colon, causing a possible chronic inflammatory mucosal damage, stimulating cancer stem cells and/or recruiting bone marrow-derived stem cells and affecting oncogenes and immune surveillance processes, may be involved in the sequence: transformation of colon epithelium to adenoma – moderate-severe dysplasia – CRC development and progression.
- Eradication of the bacterium may have an inhibiting effect on colon tumorigenesis.

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EXPERT OPINION

Current options for the treatment of *Helicobacter pylori*

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Indroduction: Treatment of *Helicobacter pylori* (*H. pylori*) infection is crucial for prevalent disease's management, including gastritis, peptic ulcer and gastric cancer, whereas novel extradigestive causal associations are increasingly being recognized. Despite long-standing efforts, there is not as yet an optimal empirical therapy to eradicate *H. pylori.*

Areas covered: In the present article the authors review current options for *H. pylori* eradication. Advantages and disadvantages of each of the recommended regimens, and the perspectives for their rational use in clinical practice, are critically discussed.

Expert opinion: The continuous rising of antimicrobial resistance has accounted for the declined efficiency of standard triple therapies, yielding < 70% eradication in most countries. Alternative first-line strategies have been proposed and largely validated and are now replacing standard-of-care therapies in areas with a high incidence of clarithromycin-resistance (> 20%). Such treatments include the bismuth-containing quadruple therapy, concomitant, sequential and levofloxacin-based regimens, the later mainly designated, together with rifabutin-based therapies as second-line/rescue options. Clinicians should be aware of the local resistance pattern and maintain first-line eradication to levels > 90% (per-protocol efficacy). This will prevent both exposing the patient to repeated treatments and spreading of secondary antimicrobial resistance. In the future, perspectives of tailored therapy and a prophylactic vaccine will obviate any treatment concern.

Keywords: antibiotic resistance, bismuth-containing quadruple, concomitant, eradication therapy, *Helicobacter pylori*, sequential

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1. Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative, helix-shaped, microaerophilic bacterium infecting up to 50% of the adult Western world's population [1]. Given its specific microbiological features, allowing survival in the acidic environment and gastric colonization, *H. pylori* has been recognized as a major human pathogen over the last decades. Shortly following its first identification in 1982, *H. pylori* infection has been associated to prevalent gastrointestinal disorders, including gastric inflammation and peptic ulcer disease [2]. Further evidence showed a causal relationship between *H. pylori* infection and neoplastic digestive diseases, such as gastric cancer and mucosa-associated tissue lymphoma, classifying *H. pylori* as group I carcinogen by the International Agency for Research on Cancer [3]. More recently, a miscellany of extragastric [4] and even extradigestive conditions [5], including hematological (idiopathic thrombocytopenic purpura, unexplained iron deficiency anemia), cardiovascular (ischemic heart disease) and neurological disorders (stroke, Parkinson's disease and Alzheimer's disease) have been associated with *H. pylori* infection, even though a clear therapeutic link with some of these conditions remains to be established.

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Article highlights.

- In recent years, increasing antibiotic resistance has accounted for the declining efficacy of standard first-line treatments (i.e., standard triple therapies), which are now reserved for low CAM-resistance areas (< 20%).
- To minimize need for repeated treatments and spreading of secondary antimicrobial resistance, infection with *H. pylori* should be approached and treated as any other infectious disease: treatment success is expected to be near 100%.
- In keeping with this change in paradigm of treating *H. pylori*, a number of new treatment strategies achieving > 90% in PP eradication have been proposed and largely validated. Such treatments comprise the 'bismuth-containing quadruple', 'sequential' and 'concomitant' regimens which are now designated by the recent Maastricht IV consensus report as preferred first-line treatment options in areas with high CAM resistance (> 20%).
- For 5 – 10% of patients in which first-line treatments are expected to fail, selection of second-line/salvage strategies should target to exclude the key antibiotic used initially, with BQT, levofloxacin- and rifabutin-based regimens representing reasonable options.
- Despite BQT, levofloxacin- and rifabutin-based regimens being valuable third-line empirical treatments, it is generally recommended that antibiotic-susceptibility testing is carried out after two consecutive treatment failures, thus, enabling choice of the optimal drug combination.
- Adjunct use of probiotics, most commonly *S. boulardii* or *Lactobacillus* spp., has gained interest in recent years as an encouraging approach to increase eradication rates and decrease treatment-related side effects.

This box summarizes key points contained in the article.

In parallel with growing recognition of its pathogenicity, the ability to reliably eradicate *H. pylori* has become a crucial aspect for prevalent diseases' management, including gastritis, peptic ulcer disease and gastric cancer [6-8]. Currently, indications for *H. pylori* eradication have been extended to conditions outside the gastroduodenal pathology: idiopathic thrombocytopenic purpura, iron deficiency anemia and vitamin B12 deficiency [9]. New treatment indications are expected to be established, as more evidence will probably be accumulated on a possible therapeutic link between *H. pylori* and extragastric disorders.

Historically, several antimicrobial agents (including clarithromycin (CAM), amoxicillin, metronidazole (MZN), tinidazole, tetracycline and the fluoroquinolones), have been shown to be effective against *H. pylori* and developed regimens (with the addition of an antisecretory agent/bismuth) have been tailored in various parameters (dosage, dosing intervals and treatment duration) in order to achieve the best outcomes in terms of efficacy/tolerability. Despite continuous efforts, the optimal empirical regimen remains to be established. Multiple factors are making infection with *H. pylori* so challenging: i) development of resistance to antibiotics, ii) the 'inoculum' effect produced by the large number of bacteria, iii) the protective effect of the thick gastric mucus layer, iv) the intracellular location of many bacteria and v) presence of multiple strain infection [10]. Moreover, individual patient's factors, such as compliance to treatment, age < 60 years, type of gastritis, presence of non-ulcer dyspepsia, CYP 2C19 polymorphisms (affecting proton pump inhibitor [PPI] metabolism), smoking and increased body mass index, have been shown to adversely affect eradication [11-13].

Hence, the present article is aimed to critically review current options for *H. pylori* eradication. Therefore an attempt has been made to review advantages and disadvantages of proposed anti-*H. pylori* regimens, and also to provide existing evidence for their clinical validation and widespread use in routine practice.

2. *H. pylori*: increasing drug resistance and declining (standard) treatment efficacy

More than a decade ago, combination of a PPI b.i.d., CAM 500 mg b.i.d. and amoxicillin 1,000 mg b.i.d. or MZN 500 mg (or 400 mg in England) q.i.d., all given for 7 – 14 days (i.e., standard triple therapy) yielded high efficacy providing eradication of 90% or more [6,14]. Given the optimal safety profile and relative simplicity, both accounting for excellent patient compliance, standard triple therapies have represented the backbone of *H. pylori* eradication worldwide [7,8].

However, in successive years, treatment efficacy with standard regimens has steadily declined: from > 90% in the 1990s, disappointing rates of < 70% are observed nowadays [15,16]. These fall short of the 80% traditionally delineated as limit for acceptability under Maastricht consensus, and are far lower than what it should be expected for an infectious disease [15]. The unacceptable performance of legacy triple therapy has been outlined by large, double-blind randomized studies [17] and meta-analyses [18]. This decline in the eradication rates is in parallel with a progressive increase in the prevalence of antimicrobial resistance, in particular resistance to CAM. Indeed, by using pooled data from 20 studies (1,975 patients), the eradication rate yielded by legacy triple therapy over CAM-resistant strains was only 18% [19], with presence of CAM resistance accounting for a decrease of almost 60% in the performance of standard regimens [10]. Indiscriminate use of CAM for infectious diseases other than *H. pylori* (mainly upper respiratory tract infections) represents the main reason for the increasing incidence of CAM resistance. The global antibiotic resistance of *H. pylori* has been reported to 17.2% for CAM and 26.7% for MNZ [20], even though the latter is considered to be of secondary importance for the success of standard regimens. Resistance to amoxicillin is exceptional and generally do not represent a substantial issue. Within Europe, rates of CAM resistance range from a highest of 49% in some areas of Spain

to a lowest of 1% in the Netherlands. As a general rule, a tendency toward lower prevalence of CAM resistance is observed in northern (vs southern) European countries (resistance rate < 10%), probably reflecting more stringent Northern policies for antibiotic use [21]. Due to this variability, attempts for international standardization of *H. pylori* treatments yielded limited success.

The progressive decline in the performance of standard therapies was indirectly reflected in European consensus recommendations. This was first by the adoption of a 'cumulative' approach, introducing second-line therapies in patients who failed to eradicate *H. pylori* (Maastricht II [7]), and successively by the definition of a regional threshold of CAM resistance (15 – 20%) at which the antibiotic should not be used if culture was not performed (Maastricht III [8]). Obsolescence of standard therapies for high CAM-resistance areas is now clearly amended in the 2012 Maastricht IV/ Florence consensus report: a threshold of 20% is used to separate the regions of high/low CAM resistance, with CAM-containing regimens maintaining their role as standard therapies only if local resistance to this agent does not exceed 20% [9]. Critically, use of standard therapies is likely to further decline, as the threshold at which CAM-based treatments lose their efficacy seems to be even lower than 20%, and may be 10% or less [22]. Efforts to improve standard regimens by increasing treatment duration [23], increasing PPI dose [24], or adding an adjuvant agent [25], yielded limited benefit to date.

3. Treatment as an infectious disease: the new paradigm in *H. pylori* eradication

H. pylori causes a serious infectious disease with a high morbidity and a huge economic burden. For many years, infection by *H. pylori* has been managed by using the same approach as for any other gastrointestinal disease: in the absence of an optimal treatment, the best available therapies are offered consecutively, delineating three (or more) treatment lines in a 'cumulative' eradication approach. The latter has been outlined by both the Maastricht II [7] and Maastricht III [8] recommendations. Under the same guidelines, a treatment was considered locally acceptable if achieving > 80% eradication rate on intention–to-treat (ITT) basis.

In successive years, this approach has been increasingly challenged by the reduced efficacy of standard therapies, with patients often requiring more treatment courses to eradicate the pathogen [26]. This resulted in: i) exposure to the adverse effects of repeated therapies and ii) development of secondary antibiotic resistance as the result of failed therapeutic attempt. Given these major shortcomings, relevant authorities have proposed that *H. pylori* should be approached and treated as any other bacterial infectious disease (for whom treatment success is expected to be near 100% [i.e., at least 95%]), aiming to decrease as possible the number of eradication failures [27]. However, in contrast to what is common practice for other infectious diseases (where selection of the optimal therapy relies on pretreatment susceptibility testing), endoscopy is not always feasible and well tolerated by all patients and is associated with a high economic burden. Hence, highly effective empirical therapies, achieving a per-protocol (PP) eradication rate of at least 90%, and probably at least 95%, are needed in order for the field to move forward [28]. This change in paradigm for *H. pylori* eradication has yielded increasing consensus among experts and finally accepted by the latest Maastricht IV consensus [9]: cure rates offered by triple treatment are less than the 80% rate aimed for at the beginning (Maastricht I – III), and below what now should be expected by treating *H. pylori* as an infectious disease.

In keeping with the perception of treating *H. pylori* as any other infectious disease, a number of new treatment strategies achieving > 90% PP eradication rates have been proposed and largely validated in recent years. Importantly, such therapies comprising 'bismuth-containing quadruple', 'sequential' and 'concomitant' regimens are now incorporated in the recent Maastricht IV consensus report, replacing standard triple therapy as preferred first-line empirical treatments in areas where prevalence of CAM resistance is high (> 20%). Advantages and disadvantages related to the use of these novel regimens are discussed below.

4. First-line treatment options yielding a PP eradication > 90%

4.1 Bismuth-containing quadruple therapy

For many years, a recommended therapeutic approach has been the addition of a PPI to the old regimen comprising a bismuth salt, tetracycline and MNZ [8]. This regimen currently represents an alternative to standard triple therapy in areas with a low CAM resistance, the main first-line therapeutic option for areas with a high prevalence of CAM resistance, and also a valid second-line regimen when legacy triple therapy fails in a low CAM-resistance setting [9]. Working independently from CAM resistance and the limited clinical impact of MNZ resistance, which can be largely overcome by increasing MNZ dose and prolonging duration of treatment (10 – 14 days), are its main advantages [16].

In an early meta-analysis, first-line use of bismuth-containing quadruple therapy (BQT) given for 10 – 14 days yielded encouraging results eradicating 90 – 100% of *H. pylori* infections in presence of CAM resistance and > 85% in areas with a high MNZ resistance [29]. This increased performance of BQT has been mainly attributed to the efficacy against MZN-resistant *H. pylori* strains, which overcome the eradication achieved with legacy triple therapy in presence of CAM resistance [30]. Even though BQT is known to be a useful alternative in a setting of high CAM resistance (average eradication of 77% after failure of triple therapy, based on pooled data analysis [31]), more recent data suggested suboptimal eradication

rates. Indeed, in a 2010 meta-analysis evaluating both BQT and standard therapy, 78.3% of the patients who received quadruple and 77% of those who received standard therapy achieved ITT eradication, indicating similar (and suboptimal) therapeutic effectiveness for both regimens [30]. In Greece, BQT has been mainly used as a second-line option with contradictory results [32,33]. In a randomized comparison, either BQT or standard treatment, both given for 10 days showed suboptimal performance (ITT: 78 and 65%, respectively), with more adverse events in the BQT group [34].

A major shortcoming of BQT is given by the non–availability of either tetracycline or bismuth salts in some countries. Attempts to replace tetracycline with doxycycline or amoxicillin yielded disappointing results [35,36]. Contrarily, skepticism regarding the large number of drugs (four at the same time) was narrowed when BQT was used in a patient-friendly formulation of a three-in-one monocapsule, containing either bismuth or both MNZ and tetracycline (licenced as Pylera®, containing a lower, 1.5 g instead of 2 g, tetracycline dose) [37]: three of these capsules are given four times daily in combination with a PPI b.i.d. for 10 days. Use of the monocapsule has been associated with improved eradication rates, reportedly exceeding 90% [16,38,39].

4.2 Sequential therapy

Proposed by Italian investigators, this regimen has been postulated as an efficient alternative to standard triple therapy and has been already included in recent consensus report as a valid option when local CAM resistance (> 20%) precludes use of standard treatments [9]. Contrary to BQT, it uses the same drugs administered in conventional treatments. It involves a simple dual regimen including a PPI plus amoxicillin for the first 5 days followed by a triple regimen including a PPI, CAM and a nitroimidazole (MNZ or tinidazole) for the following 5 days [40]. It represents the most extensively validated novel treatment option with different meta-analysis of randomized studies and one pooled data analysis available [18,41-44].

Performance of the sequential therapy has been proved superior to legacy triple therapy in a recent meta-analysis of 15 randomized controlled trials (RCTs) including 3,346 patients (91.7%, 95% Confidence Interval [CI]: 90 – 93% vs 76.7%, 95% CI: 75 – 79%, ITT analysis) [40]. Sequential therapy successfully eradicated 75% of CAM-resistant strains, although the low number with CAM resistance in the included studies (n = 4) may be the main criticism. The ability to eradicate CAM-resistant strains has been demonstrated by the optimal cure rates (exceeding 90%) obtained in studies conducted in countries with a high incidence of CAM resistance [15]. Similarly, the sequential regimen has been suggested as superior to legacy triple therapy over MNZ resistance [40,44]. Despite increased efficacy against monoresistant strains, a major shortcoming may be represented by the reduced performance in presence of dual antibiotic resistances (CAM and imidazole) [45,46].

The working mechanisms at the basis of the improved eradication rates with sequential therapy remain to be fully elucidated. It has been hypothesized that the initial administration of amoxicillin may cause a disruption of the bacterial wall preventing the development of efflux channels for CAM, which are known to rapidly transfer the drug out of the bacterial cell [47]. However, there is still uncertainty on whether the improved effect may be attributed to the sequential administration itself or simply to the larger number of antibiotics to which the microorganism is exposed. In this latter scenario, sequential administration would represent an unnecessary complexity.

Indeed, although adherence to treatment was excellent in the context of clinical trials, the complexity of requiring the patient to switch from a dual to a triple therapy halfway through could inherently interfere with compliance when this treatment is prescribed in a real clinical setting [48]. Furthermore, it should be stressed that sequential therapy has been largely experimented in Italy, where a therapeutic benefit of at least 10% (in comparison to standard therapy) has been shown [42]. However, studies conducted outside this country have questioned both optimal performance rates and the superiority of the sequential therapy as compared to legacy triple therapy [49,50]. This was also the case of a recent multicenter South American randomized trial, demonstrating standard 14-day triple therapy to be more efficacious than the 10-day sequential regimen [51]. Further validation trials, preferably conducted outside Italy, are required.

4.3 Concomitant therapy

Concomitant therapy (as sequential therapy), is now designated as a preferred first-line treatment option where high CAM resistance is present and BQT is not locally available [9]. It involves the concurrent administration of all three antibiotics used in standard therapies (amoxicillin, CAM and MNZ) given together with a PPI, all twice daily, for at least 10 days [52]. From a historical perspective, development of the concomitant treatment goes back over a decade, when in 1998 investigators from Germany and Japan proposed a short-term (3 – 5 days), four-drug, three-antibiotic regimen allowing for high efficacy (ITT eradication rates of 89 – 94%) [53,54]. It has reappeared today, but with prolonged 10-day duration, as a valid, simple and widely available first-line treatment option [55].

Several RCTs [53,56-60] have demonstrated that concomitant therapy is more effective but equally well tolerated as standard triple therapy. In a seminal meta-analysis by Essa *et al*. (9 published studies, 10 treatment arms, 771 patients, between 1998 and 2002), the pooled eradication with concomitant therapy was 89.7 and 92.9% in ITT and PP analysis, respectively, whereas in the same study, meta-analysis of five RCTs comparing concomitant against standard therapy revealed superiority of the quadruple regimen with a pooled difference of about 11% [61]. In a more recent update of this analysis, the ITT eradication rate was 90% for the concomitant versus

78% for conventional therapy [52]. Critically, most of the data comes from over a decade ago, and thus may not be valid today. Nonetheless, it should be stressed that duration of treatment in most of these previous studies was low (3 – 7 days), and importantly, a tendency toward better results has been observed with longer treatments [52]. Despite lack of direct comparison studies (except for one [62] showing a non-significant difference between 10-day [eradication success: 96%] and 5-day [90%] treatment), shorter treatment durations (3- and 5-day treatment) may be not effective today, thus prolonging treatment to 10 days may be required for optimal cure rates.

We have recently evaluated efficacy and tolerability of a 10-day concomitant regimen in Greece, a country with high resistance rates to both CAM (almost 25%) and MNZ (about 40%) [21,63]. This was first in an open-label, single-arm trial [64] and thereafter in a RCT investigating comparison to legacy triple therapy of same duration (10 days) [56]. Performance of the concomitant regimen in terms of ITT analysis was 90% in the former and 90.2% (vs 73.8% for standard triple) in the later study. Despite almost one-third (30.9%) reported at least one adverse event (most commonly taste alterations, abdominal discomfort and nausea), side effects were of mild/moderate severity, not interfering on the patient's adherence to treatment [56]. This is consistent with previous meta-analytical data [61] with shorter treatment durations (mild/moderate side effects in 27 – 51% of patients), showing prolongation of treatment to 10 days is a reasonable strategy in terms of efficacy:safety ratio [55]. A main advantage of the concomitant therapy may be represented by its efficacy against dual-resistant (CAM and MNZ) strains where the sequential therapy is reportedly more likely to fail; in a comparative study, patients with dual antibiotic resistance had significantly lower eradication rates after sequential therapy (dual resistance present vs absent: 33.3 vs 95.1%, $p < 0.0001$), but not after concomitant therapy (75 vs 92.4%, respectively, $p = 0.22$) [46]. However, criticism may arise on these results, as the study has been conducted in a low CAM-resistance setting (i.e., in Taiwan) where even standard regimens are still yielding excellent eradication. In a report from Spain, a country with high rates of antibiotic resistances, a 10-day concomitant therapy successfully eradicated 100% of CAM-resistant and 75% of dual-resistant strains (vs 75 and 60%, respectively with sequential), although the small number of CAM- and dual-resistant strains (5 and 4, respectively), precludes definitive conclusions [65]. Similarly, according to our recent (yet unpublished) data from a prospective trial (E-MACH study) conducted in the P. Faliron Hospital (Athens, Greece; recruitment period: 2010 – 2012), a 10-day concomitant therapy was successful in eradicating 7 of 10 dual-resistant *H. pylori* strains (i.e., the larger series of dual-resistant strains treated with concomitant therapy so far [46,53,54,65]).

Further advantages of the concomitant therapy include its simplicity (addition of a nitroimidazole to standard treatment) and its wider geographical validation (including Japan, Colombia, Taiwan, Spain, Greece, etc.) in comparison to sequential therapy. More robust assessment, preferentially conducted in a setting of high CAM- and dual antibiotic resistances, is required to clarify the topic of concurrent versus sequential administration.

4.4 Hybrid therapy

In a recent report, Hsu *et al.* proposed a two-step hybrid (dual-concomitant) therapy consisting of a dual therapy with a PPI (standard dose, b.i.d.) and amoxicillin (1 g b.i.d.) for 7 days, followed by a concomitant quadruple therapy with a PPI (standard dose, b.i.d.), amoxicillin (1 g b.i.d.), CAM (500 mg b.i.d.) and MNZ (500 mg b.i.d.) for another 7 days [66]. The novel regimen was demonstrated with high eradication rates (97% by ITT and 99% by PP analysis), also yielding high efficacy against *H. pylori* strains harboring dual antibiotic resistance (CAM and MNZ) for whom sequential therapy is known to be less effective. Given no substantial benefits seem to be obtained by prolonging sequential therapy from 10 to 14 days [67], the 14-day exposure to amoxicillin is probably the key for the improved effect with hybrid therapy. Notably, in a preliminary communication, hybrid therapy has been proven equally effective to a 14-day concomitant treatment (ITT eradication: 90 vs 92%, respectively, $p = 0.48$) while being associated with less occurrence of adverse effects (48 vs 68%, respectively, $p = 0.015$) [68]. It, therefore, represents a promising first-line treatment option which merits adequate consideration in future comparative studies.

4.5 Levofloxacin-based therapies

Levofloxacin, a broad-spectrum fluoroquinolone agent, has been postulated as an efficient alternative to CAM either for triple, quadruple and sequential regimens. The eradication rates of levofloxacin-based triple therapy (comprising a standard dose PPI, levofloxacin 500 mg and amoxicillin 1g, all b.i.d., for 10 days) ranged from 72 to 90% (ITT analysis) and this regimen has been suggested as an efficient alternative in settings of CAM resistance > 15 – 20% and quinolone resistance < 10% [69]. In a high CAM-resistance setting (20%; but only 3.7% of quinolone resistance), a levofloxacin-based sequential regimen was shown to be more effective than CAM-based sequential therapy [70], and this was confirmed in a recent pooled data analysis [71]. Lastly, a 5-day levofloxacin-based concomitant treatment has been suggested as equally effective and well tolerated to10 days levofloxacin-based sequential regimen, while being less expensive [72]. Despite these satisfactory results, early development of quinolone resistance (which may create difficulties in controlling respiratory and urinary tract infections), and the high incidence of adverse effects, generally discourages first-line use of levofloxacin-based regimens, which currently reserved as second-line/salvage treatments.

Table 1. Overview of recommended regimens for *H. pylori* eradication.

Treatment	Regimen	First-line treatment		Second-line/ rescue treatment
		High* CAM-resistance area	Low CAM-resistance area	
Standard triple therapy	A PPI (standard dose, b.i.d.), amoxicillin (1 g, b.i.d.) and CAM (500 mg, b.i.d.) for 14 days		✓	
BQT	A PPI (standard dose, b.i.d.), bismuth (standard dose, q.i.d.) tetracycline (500 mg, q.i.d.) and MZN (500 mg, q.i.d.) for 10 – 14 days	✓	✓	✓
Sequential therapy	A 5-day dual therapy with a PPI (standard dose, b.i.d.) and amoxicillin (1 g, b.i.d.) followed by a 5-day triple therapy with a PPI (standard dose, b.i.d.), CAM (500 mg, b.i.d.) and MZN (500 mg, b.i.d.)	✓		
Concomitant therapy	A PPI (standard dose, b.i.d.), CAM (500 mg, b.i.d.), amoxicillin (1 g, b.i.d.) and MZN (500 mg, b.i.d.) for 7 – 10 days	✓		
Hybrid therapy	A 7-day dual therapy with a PPI (standard dose, b.i.d.) and amoxicillin (1 g, b.i.d.) followed by a 7-day quadruple therapy with a PPI (standard dose, b.i.d.), amoxicillin (1 g, b.i.d.), CAM (500 mg, b.i.d.) and MZN (500 mg, b.i.d.)	✓		
Levofloxacin-based triple therapy	A PPI (standard dose, b.i.d.), levofloxacin (500 mg, b.i.d.) and amoxicillin (1 g, b.i.d.) for 10 days	✓‡		✓
Rifabutin-based triple therapy	A PPI (standard dose, b.i.d.), rifabutin (150 mg b.i.d.) and amoxicillin (1 g b.i.d.) for 14 days			✓

*Local incidence of CAM resistance > 20%.

‡First-line use of levofloxacin-based regimens might be disputed by the rapid development of levofloxacin resistance in many countries.

PPI: Proton pump inhibitor.

5. Second-line and rescue therapies

5.1 Bismuth-containing quadruple therapy

After failure of standard triple therapy, use of BQT has been generally recommended as an optimal second-line eradication strategy in previous and current consensus reports [8,9]. Second-line efficiency of BQT is highlighted either by its ability to work independently than CAM resistance and partially overcoming it to MNZ (as discussed earlier in this review), with several studies reporting satisfactory results. Based on the results of 30 studies, the weighted mean eradication rate yielded by BQT following failure of standard triple therapy was calculated to 77% (ITT analysis) [73]. However, treatment duration, which seems to be a major determinant of therapeutic efficacy [74], was variable and was 7 days in most (19/30) of these studies. Dose of MNZ is another critical factor. Notably, in the study reporting a lowest of 57% of second-line efficacy with 7-day BQT, a low MNZ dose of 250 mg q.i.d. was used [75]. Contrarily, use of a 10-day, high-MNZ dose (i.e., 500 mg q.i.d.) BQT was shown to be effective (79.7 and 90.8% in ITT and PP analysis, respectively), and comparable to a levofloxacin-based quadruple regimen, in a high antibiotic-resistance area [76]. Administrating BQT after failure of standard triple therapy is more effective [77], or at least more cost-effective [77,78], than the inverse strategy. Interestingly, Lee *et al*. recently showed that a retrial of BQT may be worthy for patients who fail to respond to second-line use of that same regimen (PP eradication: 76%) [79]. Future studies should further assess effectiveness and tolerability of a second-line/rescue, high MZN dose, of at least 10-day duration BQT regimen, preferably by using the monocapsule formulation.

5.2 Levofloxacin-based therapies

Under the recent Maastricht IV, a levofloxacin-based triple therapy represents the main second-line treatment when legacy triple therapy (in areas with a low CAM resistance) or BQT/sequential/concomitant therapies (if local CAM resistance is high) fail to eradicate *H. pylori* [9]. Evidence for second-line efficacy of this regimen has been provided by randomized trials [80] and meta-analytical data [81,82] (mean eradication rate 80% [81]), whereas a stable efficacy over time (2006 – 2011) has been recently outlined by a Spanish study [83]. In the meta-analysis by Gisbert and Morena, better results were observed with second-line use of levofloxacin-triple therapy (81% vs BQT: 70%), although statistical significance was only reached by excluding a single outlier study reporting a lowest (63%) success rate [81]. In a recent Italian

Table 2. Advantages and disadvantages of proposed regimens for *H. pylori* eradication.

Regimen	Advantages	Disadvantages
Standard triple therapy	Widely used/standard regimen Simplicity	Ineffective for high CAM-resistance areas
BQT	Working independently from CAM-resistance Largely overcoming MNZ-resistance High eradication rates using a patient-friendly monocapsule formulation Suitable for patients with penicillin allergy Well-documented second-line efficacy after standard triple therapy failure	Large number of pills (13 pills/day) Non-availability of tetracycline and/or bismuth salts in some countries Relatively high rate of side effects Prolonged treatment duration (10 – 14 days) and high MNZ-dose (500 mg t.i.d.) are required to overcome MNZ-resistance
Sequential therapy	Probably effective in high CAM-resistance settings Most widely evaluated as novel first-line treatment option	Probably reduced efficacy against dual-resistant CAM/MZN strains Complexity of switching from a dual to a triple therapy at midpoint Efficacy has been questioned in studies outside Italy
Concomitant therapy	Probably effective in high CAM- and MNZ-resistance settings Constant good results in studies conducted across different countries.	More assessment in settings with high CAM- and dual-resistances is still required Larger number of pills as compared to sequential therapy
Hybrid therapy	Probable effective in high CAM-resistance settings	Few data available on its efficacy/safety Complexity of switching from dual to concomitant therapy at midpoint
Levofloxacin-based triple therapy	Working independently from CAM- and MNZ-resistance Suitable for patients with penicillin allergy (PPI/levofloxacin/CAM regimen) Effective second-line/salvage treatment after failure of CAM- and/or MNZ-based first-line regimens	Ineffective if local quinolone-resistance> 10% Rapid development of secondary levofloxacin-resistance
Rifabutin-based triple therapy	Working independently from CAM- and MNZ-resistance	Development of mycobacterium resistance Suboptimal safety profile (recommended as third-or more-line rescue therapy)

CAM: Clarithromycin; MNZ: Metronidazole.

study, a second-line levofloxacin-triple regimen successfully treated 75% of patients with previous failure to sequential therapy and a cumulative 97.8% of eradication was achieved (PP analysis) [84]. Contrarily, no clear benefit emerged with levofloxacin-based rescue regimens (vs BQT) in two Asian studies [85,86]. Albeit third-line treatment is currently recommended to rely on antibiotic susceptibility testing [9], levofloxacin-triple regimen may be also implemented (in low quinolone-resistance areas) as an empirical salvage therapy, after both standard triple regimen (first line) and BQT (second line) have failed and culture-guided therapy is not feasible.

Optimal treatment duration and levofloxacin dosing are both an interesting issue. Di Caro *et al.*, showed 10-day levofloxacin-triple regimen was better than 7 days [87], and the same applies when using this regimen as rescue therapy [81]. Prolongation to 14 days showed second-line eradication > 90% (PP analysis), although a marked decrease was observed in presence of quinolone resistance [88]. In contrast to MNZ resistance which can partially overcome the increasing MNZ dose, it seems there is not a dose-dependent effect in overcoming levofloxacin resistance [86]: a single daily dose of the drug yielded less side effects as compared to twice daily administration without impacting eradication rates [87]. Finally, there has been little evaluation to date of a second-line levofloxacin-containing sequential therapy, showing contradictory results [71].

5.3 Rifabutin-based therapy

Rifabutin is a classic antituberculosis agent with good *in vitro* activity against *H. pylori*, even over CAM- and MNZ-resistant strains [89]. Administered in a triple regimen with amoxicillin and a PPI, all given twice daily, it represents an encouraging salvage strategy after multiple (at least two, and usually three or more) failures with key anti-*H. pylori* antibiotics. In a meta-analysis by Gisbert *et al.*, the eradication rates for second- (223 patients), third- (342 patients) and fourth/fifth-line (95 patients) rifabutin-based therapies were 79, 66 and 70%, respectively, with incidence of rifabutin-resistance (11 studies, 2982 patients) ranging from 0.6% in treatment-naïve patients to 1.59% when post-treatment patients were considered [90]. However, in a recent prospective study (100 patients) evaluating rifabutin treatment as a fourth-line option (following unsuccessful use of a sequence

of standard triple, BQT and levofloxacin-triple regimens), eradication rates where lower: 52 and 50% in PP and ITT analysis, respectively [91]. The optimal treatment duration remains to be established, but most studies suggested 10 – 12 days. A 300 mg/day rifabutin has been used more frequently and seems more effective than 150 mg/day. Occurrence of adverse events (22%, 19 – 25%) [90], with myelotoxicity being the most significant, together with concern arising in the development of resistance to mycobacterium species are the main reasons for which rifabutin-based therapies should be used as third-or-more-line rescue treatments only.

6. Patients with penicillin allergy

Presence of drug allergy to penicillin is a critical factor influencing the choice of anti-*H. pylori* therapies. Use of standard treatment, including a PPI/CAM/MZN combination, is now yielding low eradication rates [92] leading to high rates of secondary resistance [93] and hence should not be recommended unless for areas with low CAM resistance [9]. A first-line regimen comprising PPI/tetracycline/MNZ as well as the BQT, followed by a levofloxacin-containing regimen (together with a PPI and CAM) as a second-line option [92], are representing reasonable therapeutic options for this patient group.

7. Role of probiotics

Adjunct use of probiotics, most commonly *Saccharomyces boulardii* or *Lactobacillus* spp., has attracted attention as an alternative approach to increase eradication rates and decrease treatment-related side effects. The exact actions of probiotics in eradicating *H. pylori* remain largely unknown. Probable mechanisms include production of an inhibitory substance, competition for adhesion and improvement of *H. pylori*-related immune cascade, whereas restoration of the normal gut microflora may be a major determinant to prevent occurrence of side effects, especially diarrhea [94]. Evidence of an encouraging increase in the eradication rates of standard triple therapy by using adjunct *S. boulardii* (OR = 0.46; 95% CI: 0.3 – 0.7) [95] or *Lactobacillus* spp. (OR = 1.78; 95% CI:1.2 – 2,6) [96] supplementation has been provided by meta-analytical data.

8. Expert opinion

Treatment of *H. pylori* infection is now challenged by the declining competence of standard triple therapies, leading to a substantial need for novel, more efficient, first-line strategies. Prompted by the novel conception of 'treating as an infectious disease', clinicians are now aware of *H. pylori* eradication as a 'hit or miss' process, aiming to decrease as possible the sequelae related to repeated treatment courses (i.e., exposing the patient to a wide range of side effects and development of secondary antibiotic resistance). In the light of these changes, and in the 5-year period since the last Maastricht III European consensus, a significant move forward has been made in the field.

An overview of currently recommended therapeutic options is shown in **Table 1**, with the individual advantages and disadvantages summarized in **Table 2**. Efficient regimens (i.e., at least 90% PP eradication) are now available and largely validated and are designated by the updated Maastricht IV report as preferred first-line treatment options in settings of CAM resistance > 20%. These include the BQT, sequential and concomitant regimens. For 5 – 10% of patients, even these improved first-line therapies are expected to fail. In such cases, selection of a second-line treatment should target to exclude the key antibiotic initially used: if a CAM-based regimen was first used, a second-line MNZ-based regimen (such as the BQT) may be a reasonable second-line option, and then a levofloxacin-based treatment may be reserved as a third-line rescue therapy. Alternatively, a levofloxacin-based triple therapy may be employed in countries where bismuth salts are not available and in patients initially treated with BQT or novel non-BQT treatments. In this last case, BQT may be still valuable as a third-line option. Despite good results obtained by using BQT, levofloxacin- and rifabutin-based regimens as empirical third-line therapies, it is recommended that antibiotic susceptibility testing is carried out after two consecutive treatment failures, thus enabling choice of the optimal drug combination.

Although this last recommendation has been supported in all guideline reports up to now, it has not always yielded full consensus among the experts. Indeed, Wenzhen *et al*. found that culture-guided (standard triple) therapy was more effective and cost-saving (based on the only one paper focusing on the overall cost) than first-line empirical standard triple therapy, suggesting that antimicrobial susceptibility testing should be performed before any therapeutic attempt [97]. However, before clinicians seek to systematically perform *H. pylori* culture, they should take notice that this procedure is invasive (and thus not free from risks), expensive (mainly due to the associated endoscopy costs), not promptly available everywhere and in addition is time-consuming and do not guarantee 100% '*in vivo*' treatment success [98]. Recent introduction of highly effective first- and second-line empirical treatments (PP eradication > 90% and > 80 – 90% respectively) will likely outplace the debate of performing culture before at least two empirical treatment failures.

It should be noted that eradication rates provided in this article may be prone to significant geographical variability secondary to critically important differences in the local backgrounds of antibiotic resistance. As anti-*H. pylori* treatments are empirically given, the following two rules are important: i) clinicians should have knowledge of the local resistance rates and always prescribe what works best locally; ii) they should always confirm resolution of the infection (by post-treatment testing) and always re-treat patients who fail to eradicate *H. pylori*. This will create a useful feedback,

providing an indirect measure of the local antimicrobial resistance, and will prevent them for prescribing locally unsuccessful regimens.

In the foreseeable future, eradicating *H. pylori* infection will become increasingly important as novel therapeutic links are likely to emerge. Despite substantial progress, there is still work to be done in order for current and emerging empirical treatment strategies to be further validated and properly optimized in terms of efficacy:safety ratio. Recent changes in recommended regimens, but also in the perception of *H. pylori* infection, are highlighting transition in a new era of *H. pylori* therapy. This coincides with the end of standard triple therapies as indisputable gold standards, and their replacement by an expanded toolbox of innovative strategies, which are largely based on the same key antibiotics, but are experimented to overcome increasing antimicrobial resistance. Future research should focus on the development of noninvasive or minimally invasive tests for accurate and cost-effective characterization of *H. pylori* therapeutic susceptibility. In this setting, advances in pharmacogenomics will be of cardinal importance, and already some steps forward have been done, as for instance with CYP2C19 genotypes of *H. pylori* [99]. Pharmacogenomic-based tailored therapy is thus a fascinating perspective likely to replace empirical treatments in the future. Last but not the least, a prophylactic vaccine would represent the ideal approach to control spread in the population of *H. pylori* (i.e., a major carcinogen), obviating any treatment concerns [100]. Until then, efforts to optimize empirical therapies should continue.

Declaration of interest

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that increase the risk of GI bleeding, it also makes sense that a combination of offending drugs rather than one agent potentiates the risk further. If the authors had pooled the results for previous peptic ulcer, NSAIDs, and aspirin in Table 5, the total population attributable fractions would be 14.7%, a figure that is relatively similar to non-GI comorbidity of 18.4%.

Our opinion is that, although not undermining the importance of non-GI comorbidities, the contribution from NSAIDs and antiplatelet agents should not be disregarded totally, the effect of which may differ with different populations (current, eradicated or absence of *Helicobacter pylori* infection),[2,4] type of lesions (gastric vs duodenal), and drug combinations. The combination of comorbidities and offending drugs is likely to carry the greatest risk for sustaining nonvariceal upper GI bleeding in an elderly population.

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Conflicts of interest
The authors disclose no conflicts.

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Reply. Thank you for your letter regarding our paper. We welcome the opportunity to clarify the points raised. We agree with the authors of the letter that the contribution from nonsteroidal anti-inflammatory drugs, anticoagulants, aspirin, and previous peptic ulcers should not be ignored; indeed, our paper showed that these risk factors were all associated with bleeding. However, pooling the sequential attributable fraction (SAF) estimates for these risk factors is incorrect when risk factors are not exclusive and independent.[1,2] Recalculating the SAF for the combined effect of nonsteroidal anti-inflammatory drugs, aspirin, previous ulcers, and anticoagulants estimates a SAF of 11%. Although this reemphasizes the importance of known risk factors, it still demonstrates that nongastrointestinal comorbidities make a large contribution to bleeding in addition to known risk factors.

We also agree with the letter that the population-attributable risk of various factors varies by definition in different populations, as well as for different types of lesions. In our paper, we were able to show that the contribution of previous ulcers, aspirin, and nonsteroidal anti-inflammatory drugs to the risk of peptic ulcer bleeds was greater than for nonpeptic ulcer bleeds. There were 4823 peptic ulcers in our study; 2003 (12% of all bleeds) were gastric ulcers, 2191 (13%) were duodenal ulcers, and 480 (3%) had both recorded. An additional 1% had a code for peptic ulcer or gastrojejunal ulcer. When stratified by gastric or duodenal ulcer the SAF estimate for comorbidity was 17% and 20%, respectively. Although this shows some variation in the contribution of comorbidity by lesion location, nongastrointestinal comorbidity remained an important independent risk factor for both types of bleed.

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Is There a Nonbismuth Quadruple Therapy That Can Reliably Overcome Bacterial Resistance?

Dear Sir:

In the July issue of GASTROENTEROLOGY Molina–Infante et al reported acceptable (grade B) and high (grade A) *Helicobacter pylori* eradication rates using optimized hybrid and concomitant nonbismuth quadruple therapies, in populations with relatively high rates of antibiotic resistances (ie, Spain and Southern Italy).[1,2] This is a very important study because as yet most data concerning the efficacy of these first-line treatments were coming from low clarithromycin resistance areas.[3,4] The authors used antimicrobial susceptibility data to conclude that dual antibiotic resistance (ie, to both clarithromycin and metronidazole) did affect cure rates with the hybrid but not with the concomitant regimen. This may be true with an optimized (14-day) regimen, but, as outlined by the same authors, the small number of patients with clarithromycin-resistant (and consequently with dual-resistant) strains precludes drawing definitive conclusions.

As mentioned by Graham and Shiotani,[5] to estimate the treatment efficacy in any area with a known resistance pattern it is very important to know the exact percentage of strains with different resistance patterns cured by a specific regimen. Thus, all data, especially those regarding specific resistance patterns, must be solid and

Table 1. Efficacy of Concomitant and Sequential Nonbismuth Quadruple Therapies on Dual (Metronidazole and Clarithromycin)-Resistant Strains of *Helicobacter pylori*

Study (first author)/Journal	Year	Treatment duration (d)	Patients (cured/total)	Eradication rate (%)
Concomitant				
Treiber/Arch Intern Med	2002	5	2/4	50
Okada/Aliment Pharmacol Ther	1999	7	3/4	75
Wu/Clin Gastroenterol Hepatol	2010	10	3/4	75
Molina-Infante/Helicobacter	2012	10	3/4	75
Huang/J Dig Dis	2012	10	2/2	100
Georgopoulos/Helicobacter	2013	10	7/10	70
Molina-Infante/Gastroenterology	2013	14	3/3	100
Totals (variance of days/total patients/mean percentages)		5–14	23/31	74
Sequential				
Vaira/Ann Intern Med	2007	10	0/4	0
Romano/Gut	2010	10	0/3	0
Wu/Clin Gastroenterol Hepatol	2010	10	1/3	33
Molina-Infante/Helicobacter	2012	10	3/5	60
Huang/J Dig Dis	2012	10	2/4	50
Liou/Lancet	2013	10–14	3/8	37.5
Totals (variance of days/total patients/mean percentages)		10–14	9/27	33

based on an adequate number of patients. From this point of view, single-arm studies with availability of pretreatment *H pylori* cultures may be more useful to perform than randomized, controlled trials, because the former may more readily allow an adequate sample size by focusing on a single regimen. For example, in a recent study published by our group, dual resistance proved to be the only independent factor compromising the efficacy of 10-day concomitant nonbismuth quadruple regimen in a similar setting of high clarithromycin resistance. According to our data, based on 106 patients with available antibiotic susceptibility tests, this regimen eradicated *H pylori* in 7 of 10 patients (70%) harboring dual-resistant strains who took the regimen as first-line treatment and in only 4 of 12 (45%) of those who took it as second-line treatment after a failed standard triple therapy.[6]

Our findings are in line with previous relevant studies reporting on dual resistant strains of *H pylori* (Table 1). Overall, 5- to 14-day (mostly 10-day) concomitant treatment was successful in 23 of 31 (74%) as first-line eradication treatment. In contrast, only 9 of 27 (33%) of such strains were eradicated by the so-called sequential (10- to 14-day) nonbismuth quadruple therapy. Thus, dual resistance seems to compromise concomitant treatment but to a considerably lesser extent that the sequential one, making the former a more reasonable option in areas with a high incidence of clarithromycin and/or metronidazole resistance. Unfortunately, few data have been reported for dual-resistant strains with the hybrid therapy so far. Thus, its implementation in high resistance areas warrants further consideration.

Another point mentioned by the authors as a limitation of their study is the use of the Epsilometer test (E-test) for determination of antimicrobial susceptibility.

The E-test tends to overestimate metronidazole resistance as opposed to the agar dilution reference method, possibly resulting in misclassification of a number of *H pylori* strains as metronidazole or dual resistant.[7] In our study, we performed both tests showing very good correlation, particularly in dual-resistant strains. However, the E-test tended to overestimate metronidazole resistance, in particular when levels of drug resistance were high, but without changing the final pattern. Nevertheless, discrepancies may appear in resistance levels close to the reference point ($MIC_{90} = 8\ \mu g/mL$), possibly requiring further confirmation by agar dilution test.[8]

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The authors disclose no conflicts.

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Reply. We would like to thank Georgopoulos et al for their kind remarks on our manuscript.[1] In a similar line of work, these authors have recently demonstrated that 10-day concomitant therapy is a highly effective therapy (>90%) in Greece, where the clarithromycin resistance rate is around 40%.[2] Notwithstanding the fact that in our

gave an Ulcerative Colitis Disease Activity Index (UCDAI) score of 12. Higher doses of PSL and AZA in combination with leukocytapheresis failed to induce remission (UCDAI score: 7). Addition of immunosuppressants or biologics was deemed unfeasible due to the patient's age, impaired glucose tolerance, and old tuberculosis, as well as the significant risk of concomitant infectious disease. After ruling out the presence of ischemic heart disease, cerebrovascular disorder, or other cardiovascular or malignant disease, adrenomedullin (AM; 1.5 pmol/kg/min) was intravenously administered for 8 hours per day for 12 days. A few days after starting the AM treatment the patient's abdominal pain and bloody stool appeared to go into remission. No adverse events were observed apart from a slight decline in blood pressure. Endoscopy at 2 weeks revealed significant mucosal regeneration (Fig. 1) and spider web-like scarring in some ulcers (Fig. 2), and the patient's UCDAI score had declined to 2. After 3 months, all of the patient's colonic lesions had healed with scarring and her UCDAI score had reached 0, so the PSL was discontinued.

AM was first identified as a biologically active peptide with potent vaso-dilating action,[1] but is now known to exert a wide range of physiological effects, including cardiovascular protection,[2] neovascularization, and suppression of inflammation and apoptosis. We previously reported that AM therapy was effective in an animal colitis model,[3] and that AM's mechanism of action is likely attributable to its suppression of inflammatory cytokines and activation of regulatory cytokines in intestinal intraepithelial lymphocytes, as well as to its protection of intercellular junctions and its antibacterial activity.[4] In addition, AM reportedly suppresses cytokine production in trinitrobenzene sulfonic acid (TNBS)-induced colitis,[5] and exerts beneficial effects on microvascular function[6] and the reepithelialization[7] of ulcers in an experimental model of colitis.

Although AM has potent hypotensive activity, we observed only minor hemodynamic effects after administering a dose of 1.5 pmol/kg/min, which we considered safe based on human dose-response data in our possession.

Conventional treatment of active UC focuses on steroids, immunosuppressants, and biologics, but the use of these drugs is restricted in geriatric and immunocompromised patients.[8] AM, on the other hand, is a physiological peptide and is therefore anticipated to have excellent safety. Here we present the first reported case in which AM was used to treat a patient with intractable UC. AM treatment produced mucosal regeneration accompanied by marked neovascularization and vasodilation visible on endoscopic examination. These findings are suggestive of AM's potential to be a ground-breaking modality with a novel mechanism of action that differs from existing immunomodulation therapy.

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Potential Oncogenic Properties of Mobilized Stem Cells in a Subpopulation of Inflammatory Bowel Disease Patients Infected with *Helicobacter pylori*

To the Editor:

Marlicz et al[1] concluded that Crohn's disease (CD) triggers the mobilization of various types of stem cells, such as hematopoietic stem progenitor cells, into peripheral blood in patients suffering from this disease, while the significance and precise role of these mobilized cells in repair of damaged intestine requires further study. However, the authors did not discuss the possibility of potential oncogenic properties of the mobilized stem cells, at least in the subgroup of patients possibly infected with *Helicobacter pylori (H. pylori).*

In this regard, although relative data indicate an absence or inverse association between *H. pylori* and inflammatory bowel disease (IBD), the prevalence of *H. pylori* infection in the IBD patients appears to be 38.2%–47% in Europe.[2] Moreover, enterohepatic and gastric *Helicobacter* species have been documented in fecal specimens from children with CD using polymerase chain reaction (PCR), and *H. pylori,* for example, was recently found in the intestinal mucosa of a patient affected by CD.[3]

Experimental data indicate that *H. pylori* infection leads to development of chronic inflammation, hyperplasia, metaplasia, dysplasia, and recruitment

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and accumulation of bone marrow-derived cells (BMDCs) in the mouse gastric epithelial mucosa. Nearly 25% of dysplastic lesions include cells originating from BMDCs, thereby indicating that BMDCs can participate in preneoplastic lesions preceding gastric carcinoma development; there is a role for engraftment of circulating BMDCs, which may contribute to tumor formation in animal models with *H. pylori*-induced chronic gastric inflammatory processes,[4] thus further suggesting the possibility of the potential contribution of BMDCs in human gastrointestinal carcinoma.

In this regard, we recently conducted a pilot study using tissue sections of biopsies of human gastric cancer in which *H. pylori* was detected by Cresyl violet staining.[5] Moreover, stem cells and neovessels were detected by immunohistochemical method using a monoclonal antibody, anti-CD34; CD34, also mentioned by the authors,[1] is a surface glycoprotein expressed on hematopoietic stem cells and is used as an important marker of these cells and neovessels. In addition, cyclin D1, involved in the regulation of cell proliferation, was also detected by immunohistochemical method.[5] Other relative data indicate that *H. pylori* induced cytotoxin VacA exhibits chemotactic activities to the BMDCs and induces BMDCs to produce proinflammatory cytokines, leading to chronic inflammation with potential oncogenic consequences. Therefore, it would be reasonable to speculate that chronic *H. pylori* infection in both mice and humans induces repopulation of the stomach with BMDCs that may facilitate gastric cancer progression.[4,5] 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 also be combined with supporting observations of BMDCs in other tumors such as Barrett's esophageal adenocarcinoma, Kaposi sarcoma, cancer-associated fibroblasts, or benign and malignant tumors of the skin.[4,5]

Other relative data, using the stem cell marker CD44 (the integral membrane molecule CD44 is a marker of human hematopoietic stem and progenitor cells),[6] indicate that the CD44+ gastric cancer stem cells show the stem cell properties of self-renewal, the ability to form differentiated progeny, and, moreover, increased resistance for chemotherapy or radiation-induced cell death; *H. pylori* either directly or through a local inflammatory response is responsible for increased expression of CD44 and its variant CD44 v9,[4] thereby suggesting a possible *H. pylori* induction of CD44+ BMDCs/gastric cancer stem cells involved in gastric cancer development and progression.[4]

Because *H. pylori* also induces inflammatory changes in colonic mucosa, it would be reasonable to further speculate that chronic *H. pylori* infection in humans induces repopulation of the colon with BMDCs that might facilitate colon cancer development and progression. In this respect, our own preliminary studies indicated the presence of *H. pylori* in malignant colonic tissue in 34 of 41 (82.9%) patients with colorectal cancer (23 men, mean age 73.6 ± 7.9 years).[7] Moreover, increased expression of CD44 in malignant tissue but not in the adjacent normal colonic mucosa was noticed in 31 of 41 (75.6%) patients with colorectal cancer.[7] Extending these preliminary data we currently included 50 patients (28 men, mean age 71.3 ± 9.7 years) with colorectal cancer and 25 patients (13 men, mean age 72.8 ± 10.1 years) with colonic polyps with the following results: *H. pylori* presence and increased expression of CD44 in malignant tissue of patients were observed in 84% and 78%, respectively, confirming our preliminary data. Comparable data in adenomatous tissue of patients with colonic polyps were also observed in 64% and 16% of patients, respectively (unpubl. data). Therefore, these findings suggest the possible BMDC involvement in *H. pylori*-associated colon cancer initiation and/or progression. Finally, we found that the histologic presence of *H. pylori* in malignant colonic tissue was associated with Ki67 oncogene increased expression in all tumor specimens and low expression in all adjacent tissue specimens.[8] Moreover, p53 increased and low expression was observed in 72.5% and 100% of tissues specimens, respectively. Likewise, antiapoptotic Bcl-2 protein was observed in 60% and 9% of tissue specimens, respectively, whereas proapoptotic Bax protein was observed in 9% and 100% of tissue specimens, respectively.[8] Therefore, *H. pylori* colonizing colonic tumor tissue seems to be associated with an increased cell proliferation and impaired apoptotic process in malignant tissue compared with normal adjacent colonic mucosa, thereby further contributing to colon cancer progression.[8] Similar considerations seem to be introduced in the aforementioned subgroups of IBD patients infected with *H. pylori* infection.

However, larger-scale relative studies are needed to show that, apart from repair of damaged intestine also mentioned by the authors,[1] the BMDCs move into areas of the upper and lower gastrointestinal tract in the context of *H. pylori* chronic injury or inflammation with potential long-term malignant consequences in IBD patients tested *H. pylori*-positive.

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Pulmonary Carcinoid Tumor in a Patient on Adalimumab for Crohn's Disease

To the Editor:

Antibodies against tumor necrosis factor alpha (TNF-α) are safe and accepted treatments for luminal and fistulizing Crohn's disease (CD).[1,2] In addition to the proven efficacy of these drugs (infliximab, adalimumab, certolizumab), there are inherent risks of this immune-suppressing therapy, including infusion/injection reactions, serious infections, and hematologic malignancy. Solid organ malignancies have also been described in this immune-suppressed population. However, there are limited data directly implicating anti-TNF therapy and increased prevalence of solid malignancies. We report the first case of pulmonary carcinoid tumor developing in a Crohn's disease (CD) patient on adalimumab therapy. A question remains: Does biologic therapy need to be withheld once solid malignancy is treated with expectation of cure?

A 63-year-old prior smoker woman with penetrating ileocolonic CD with quiescent luminal disease and active perianal disease presented with perianal fistulas and a recently drained perirectal abscess. Adalimumab monotherapy maintained the patient in clinical remission for 10 months prior to her current presentation.

Computed tomography (CT) of the abdomen and pelvis revealed bibasilar pulmonary nodules, previously seen fistula, and mild thickening of the rectum. A follow-up CT scan of the chest revealed a >2 cm right upper lobe (RUL) lung mass and multiple bibasilar subcentimeter pulmonary nodules. Comparison to prior chest x-rays before exposure to anti-TNF-α therapy revealed stable pulmonary nodules but did not reveal the mass. Transbronchial biopsy and bronchoalveolar lavage (BAL) were inconclusive. Positron emission tomography (PET) revealed increased uptake in the mass and no other areas suggesting a localized malignancy (Fig. 1). A video-assisted thoracopic surgery (VATS) was performed for tissue diagnosis and staging. Pathologist review determined a typical carcinoid tumor, T1N0. Adalimumab was stopped and the patient was placed on ciprofloxacin for perianal disease. She underwent a successful and curative lobe wedge resection. Repeat CT chest 3 months after surgery showed no evidence of recurrence. The patient is currently in clinical and endoscopic remission on methotrexate weekly injections.

Increased rates of non-Hodgkin's B-cell lymphoma and T-cell lymphomas have been shown to be associated with the use of anti-TNF agents. [3]Studies have also made an association between biologics and solid tumors including skin cancer, nonsmall cell lung cancer, and breast cancer.[3,4] A possible theory regarding this association discusses the important TNF-α role for natural killer/CD8 lymphocyte cell lysis and modulating adaptive immunity, which contributes to maintaining tumor equilibrium, and suppressing TNF-α may enhance proliferation of solid organ malignancies.[5,6]

To our knowledge, this is the first case of primary pulmonary carcinoid tumor associated with anti-TNF therapy to be reported in the literature, but reports of carcinoid tumors in immunosuppressed populations have been reported. Nemes et al[6] reported a rapidly growing, ultimately fatal primary liver carcinoid associated with cyclosporine use following renal transplantation. Lito et al[7] published a series of four pancreatic neuroendocrine tumors associated with human immune deficiency

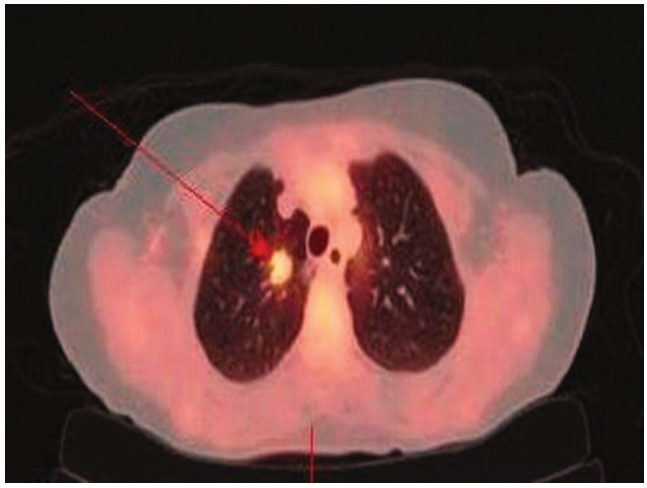

FIGURE 1. PET CTof right upper lobe lung mass. Enhancement (arrow) consistent with malignancy.

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Potential conflict of interest: Nothing to report.

Helicobacter pylori Induced Cognitive Dysfunction Might Be Associated With Falls and Fractures in Cirrhosis

To the Editor:

In their prospective series, Soriano et al.[1] concluded that cognitive dysfunction (CD) is a factor associated with falls in patients with cirrhosis, and further studies are warranted to address the mechanisms implicated in this predisposition and to design preventive strategies.

Apart from CD causes that can be the result of multiple issues, including the etiology of cirrhosis, mentioned by the authors,[1] *Helicobacter pylori* infection (*Hp*-I) appears to be a common denominator associated with CD-related falls and fractures and liver cirrhosis.[2-4] In this respect, we reported that *Hp*-I is frequently detected in neurodegenerative diseases including CD and Alzheimer's disease (AD) and *Hp* eradication may positively influence AD manifestations at 2- and 5-year clinical endpoints,[2] thereby supporting a role for this common infection in the pathobiology of the disease. Others also found that AD patients infected by *Hp* tended to be more cognitively impaired, and patients with dementia have a higher risk of falls and fractures[3]; *Hp*-I may also be responsible for osteoporosis. Moreover, hepatitis B (HBV) and C (HCV) are among the commonest causes of liver cirrhosis worldwide[4,5] and *Hp*-I is strongly associated with HBV- and HCV-related cirrhosis in Europe (Italy); *Hp*-I is more common in cirrhosis patients with hepatic encephalopathy (HE) than in those without,[5] and HE is not a fully reversible condition.

Summarizing the aforementioned data, *Hp* may be involved in the pathophysiology of cirrhosis-related CD by several mechanisms,[4] such as the release of proinflammatory and vasoactive substances, involved through blood-brain barrier (BBB) disruption, in a number of vascular disorders including AD, which can lead to long-term neurologic deficits; promoting platelet-leukocyte aggregation proposed to play pathophysiologic roles in AD and liver fibrosis; producing reactive oxygen metabolites involved in the AD pathophysiology and complications of cirrhosis; or influencing the apoptotic process, an important form of cell death in AD and cirrhosis. Finally, activated monocytes (possibly infected with *Hp* due to defective autophagy resulting in *Hp* replication in autophagic vesicles) might also enter the brain due to BBB disruption contributing to cirrhosis-related CD development associated with falls and fractures.[3]

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Reply:

We appreciate the comments of Kountouras et al. regarding our article showing that cognitive dysfunction evaluated by the Psychometric Hepatic Encephalopathy Score (PHES) is associated with falls during follow-up in patients with cirrhosis.[1] We agree with these authors that subclinical cognitive dysfunction in cirrhosis is a multifactorial issue. Although minimal hepatic encephalopathy plays a key role in such dysfunction, other factors, such as etiology of cirrhosis, comorbidities, or psychoactive treatments, can also be implicated.[1-3] Multiple factors are also involved in the risk of falling.[4]

Helicobacter pylori infection has been suggested as a factor predisposing patients with cirrhosis to overt hepatic encephalopathy and minimal hepatic encephalopathy through the increase in ammonemia[5,6] or, as proposed by Kountouras et al., through the proinflammatory state. However, this association has not been clearly demonstrated.[5] The relationship between *H. pylori* and dementia in patients without cirrhosis is also controversial.[7,8] To our knowledge, there are no studies evaluating the potential link between falls or fractures and *H. pylori* infection. In any case, we cannot study the relationship between *H. pylori* and cognitive dysfunction or falls in our study group because *H. pylori* infection was not systematically evaluated in all the patients.

As recently pointed out by Butterworth,[9] the main contribution of our article is that it shows that psychometric testing can predict the risk of falling in patients with cirrhosis, in addition to its already known ability to detect the risk of overt hepatic encephalopathy, mortality, and traffic accidents.[2,10] This finding could help to establish measures to prevent falls and fractures and their consequences in this population.[1,9]

When considering the mechanisms involved in the risk of falling, we found no correlation between the severity of PHES impairment and predisposition to falling. This suggests that the main cause of falling is not cognitive dysfunction *per se* but a coincident neuromuscular disturbance, such as parkinsonism, cerebellar degeneration, or sarcopenia.[1,9] The precise mechanisms by which patients with cirrhosis and impaired PHES have a higher tendency to fall remain to be determined.

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Disparate Profiles of Dys-Regulated miRNAs in Activated Hepatic Stellate Cells

To the Editor:

We read with great interest the article by Lakner et al. in a recent issue of Hepatology.[1] In this study, the authors found a novel regulator of hepatic stellate cells (HSCs), miR-19b, which acted via the transforming growth factor-β signaling pathway. miR-19b was expressed differently between quiescent and activated HSCs, using comparative analysis of microRNA (miRNA) expression. As is well known, comparative analysis is the gold standard approach for detecting dys-regulated miRNAs.

This same approach has been used on HSC in 4 other studies related to the topic.[2-5] The profiles of dys-regulated miRNAs in activated HSCs are summarized in Table 1. The same protocol was executed with the following steps in these studies: step 1, quiescent HSCs were isolated from normal rat liver; step 2, activated HSCs were acquired by culturing quiescent HSCs in vitro for 10 or 14 days until activated; step 3, the different miRNA expression patterns of activated and quiescent HSCs were analyzed by comparative analysis. However, there was an interesting phenomenon shown in Table 1, which was that the profiles of dys-expressed miRNAs in activated HSCs varied greatly across the studies. The issue remains why the same protocol for detecting dys-regulated miRNAs in activated HSCs resulted in such different miRNA profiles.

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LETTER TO THE EDITOR

Helicobacter pylori Infection and Insulin Resistance

To the Editor,

We read with interest the study of Naja et al. [1] which reported no association between *Helicobacter pylori* (*H. pylori*) infection and insulin resistance (IR) or metabolic syndrome (MetS) in an adult Lebanese population. More specifically, no difference was shown between *H. pylori*-specific immunoglobulin G (IgG) antibody positive (n = 160) and negative (n = 148) individuals regarding either homeostatic model of assessment—IR (HOMA-IR) or MetS rates. The authors concluded that "eradication of *H. pylori* infection to prevent IR or MetS is not warranted."

We previously conducted a systematic review summarizing the epidemiologic evidence concerning the association between *H. pylori* infection and IR quantitative only indices [2]. Seven cross-sectional studies and two nonrandomized open-label controlled trials investigating the effect of *H. pylori* eradication on IR were included in this systematic review, with HOMA-IR being used in all studies to quantify IR. There appeared to be a trend toward a positive association between *H. pylori* infection and HOMA-IR [2]. More specifically, when the study groups were divided into *H. pylori* positive or negative, higher HOMA-IR was found in all but one study. Studies suggesting negative results, as Naja et al. [1], are important, enhancing our understanding, although they are more difficult to publish, having to overcome publication bias.

In an attempt to interpret the negative results, we compared Naja et al. [1] study with those previously published. First, Naja et al. study was not specifically designed for the study of association between *H. pylori* infection and IR; specifically, it was designed for the association between dietary patterns and the risk of MetS. As a consequence, history of previous eradication treatment or recent use of antibiotics and/or proton pump inhibitors were not recorded; given that eradication treatment results in transition to *H. pylori* IgG seronegativity of some of previously seropositive patients, both parameters could have affected the frequency of seropositivity in this study.

More importantly, both *H. pylori* positive and negative groups had extremely high HOMA-IR values (5.58 ± 3.15 vs 6.74 ± 6.83, respectively), with the variability being higher in *H. pylori* negative group. This becomes more interesting when considering the relatively low body mass index (26.7 ± 4.9 vs 27.4 ± 4.9 kg/m^2, respectively) and waist circumference (87.7 ± 12.8 vs 89.5 ± 14.3 cm, respectively) of either group. It is highlighted that mean HOMA-IR values in similar previously published studies is between 0.89 and 3.89 [2]. The higher variability of HOMA-IR in *H. pylori* negative group may account for higher, although nonstatistically, rates of individuals with IR in *H. pylori* positive compared with *H. pylori* negative group (58.1% vs 49.3%, for within group percentage), when the cut-off of 2.5 was used for HOMA-IR, whereas the mean value of HOMA-IR was higher in the *H. pylori* negative group. The reason for such high HOMA-IR values in Naja et al. [1] study is not obvious; the use of insulin treatment in some of the patients, which might have affected the result, was not reported as exclusion criterion by the authors. Independently from the reason of high HOMA-IR values, it could be speculated that *H. pylori* infection may not be associated with HOMA-IR in highly insulin resistant populations.

Furthermore, it has been reported that the prevalence of *H. pylori* infection even in healthy donors in Beirut, where the study by Naja et al. was conducted, is high (68.3%) [3]. This means that to prove an association between *H. pylori* infection and IR or Mets in Beirut (recently having in Lebanon the highest estimated prevalence (31.2%) mentioned by the authors [1]), and, more importantly, to conclude that eradication of *H. pylori* infection to prevent IR or MetS is not warranted, several hundreds or even a few thousands of participants are required. Instead, Naja et al. study included only a small number of patients, and therefore the power of their study was low.

More recently, there are two more studies providing data for the association between *H. pylori* infection and HOMA-IR. In one of them, *H. pylori* seropositive individuals at enrollment were 2.7 times more likely to develop diabetes in a 10-year period than *H. pylori* seronegative ones, which remained significant after adjustment for co-founders [4]. However, in this study baseline HOMA-IR was similar between *H. pylori* positive (medians (interquartile range): 1.9 (1.2–3.1)) and negative individuals (1.8 (0.99–3.7)). Nevertheless, if HOMA-IR had been measured at the time diabetes was

diagnosed, instead of baseline, HOMA-IR values between *H. pylori* positive and negative individuals might have been significantly altered [5].

In another study with nonalcoholic fatty liver disease (NAFLD) patients, the individuals with history of *H. pylori* eradication treatment and/or *H. pylori* seropositivity had significantly higher HOMA-IR levels (mean ± standard error of the mean: 2.64 ± 0.36) compared with those without either history of *H. pylori* eradication treatment or *H. pylori* seropositivity (1.01 ± 0.19) [6]. Notably, higher rates of anti-*H. pylori* IgG titers were observed in NAFLD compared with control group.

An association between *H. pylori* infection and MetS seems to be appealing, given that almost half of the world's population is affected by *H. pylori* infection, the prevalence of MetS is increasing worldwide, and, if an association was proved, *H. pylori* eradication might have therapeutic beneficial effects on MetS-related morbidity, including diabetes and NAFLD [2]. Nevertheless, existing evidence remains insufficient either to support a solid association between *H. pylori* infection and HOMA-IR or to introduce *H. pylori* eradication for treating IR.

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Competing interests: the authors have no competing interests.

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Letter to the Editor

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Relationship between *Helicobacter pylori* infection and autoimmune disorders

Keywords: autoimmune pancreatitis; *Helicobacter pylori*; inflammatory bowel disease; multiple sclerosis.

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To the Editor,

Ram et al. [1] demonstrated a dichotomy relation between infections, specifically with *Helicobacter pylori*, and autoimmunity or autoimmune diseases, by using serology (*Hp* IgG antibodies) for the detection of *Hp* infection (*Hp*-I).

Although the serological test establishes the presence of *Hp*-I, it does not discriminate between current and old infections. Such a distinction is crucial because only current *Hp*-I induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with the host components, thereby contributing and possibly perpetuating autoimmune disorders. Moreover, eradicating *Hp*-I might alter the pathophysiology of these disorders [2, 3]. For instance, molecular mimicry of host structures by constituents (i.e., the saccharide portion of lipopolysaccharides) of *Hp* is thought to be connected with the development of autoimmune sequelae in autoimmune neuropathies, primary biliary cirrhosis (PBC) or autoimmune pancreatitis (AIP), that induce apoptotic damage of neurons, liver tissue, or pancreatic tissue [4].

By using histology, the recognized practical gold standard for the diagnosis of current *Hp*-I, our series showed a strong association between *Hp*-I and multiple sclerosis (MS) [4]. In contrast, as mentioned by the authors [1], *Hp*-I might be a protective factor against conventional MS in Japanese patients [1], by using the serological test. Apart from the latter limitation, we believe that, since *Hp* prevalence is also significantly lower in gastro-esophageal reflux disease (GERD) patients from East Asia than in those from Western countries, possibly indicating a protective role against GERD, comparable data might also explain either the likely protective role of *Hp* against MS in Asians or its causative role in MS in Caucasians [5]. Besides, although relative data indicate an absence or inverse association between *Hp* and inflammatory bowel disease (IBD), as the authors also claimed [1], the prevalence of *Hp*-I in the IBD patients appears to be 38.2%–47% in Europe. Moreover, enterohepatic and gastric *Helicobacter* species have been documented in fecal specimens from children with Crohn's disease (CD) using PCR, and *Hp* was recently found in the intestinal mucosa of a patient with CD [6]. Therefore, ethno-geographic gradients in IBD risk might also explain the dichotomy relations between *Hp* and IBD.

Apart from the diversity of autoimmune diseases mentioned by Ram et al. [1], *Hp*-I also appears to be involved in the AIP pathophysiology. In this regard, we initially proposed that *Hp* might trigger AIP through molecular mimicry [2, 4]. Specifically, as in the case of AIP, *Hp* is also associated – via molecular mimicry of host structures by its constituents – with the same autoimmune conditions like Sjögren's syndrome (SS) (i.e., autoimmune sialadenitis), PBC, primary sclerosing cholangitis, autoimmune hepatitis (AIH) or hepatitis C virus (HCV)-related liver disease which triggers autoimmune sequelae (AIH and HCV-related SS). These *Hp*-related diseases are also characterized by fibrotic changes and/or lymphoplasmacytic inflammations, accompanied by aberrations of T cell apoptosis that contribute to hepatobiliary- or extrahepatic-tissue destruction. Considering that *Hp* is involved in the pathogenesis and pathophysiology of the above-mentioned autoimmune disorders, we proposed that *Hp*

might trigger AIP through induction of autoimmunity and apoptosis [2, 4].

Prompted by this theory, Guarneri et al. [7] sought to identify the potentially cross-reactive human and bacterial protein(s) using amino acid sequence comparison. This technique has been used in the field of immune-related diseases, yielding significant results; by using in silico protein analysis and search for HLA binding motifs to verify this hypothesis, they found a significant homology between human carbonic anhydrase II, an enzyme located in the pancreatic ductal epithelium, and α-carbonic anhydrase of *Hp*, an enzyme fundamental for the survival and proliferation of the bacterium in the gastric environment. Moreover, the homologous segments contain the binding motif of the HLA molecule DRB1*0405 [7]. Notably, possession of the HLA DRB1*0405-DQB1*0401 genotype confers a risk for the development of AIP. These data strengthen our speculation that gastric *Hp*-I can trigger AIP in genetically predisposed subjects [4].

More recently, Frulloni et al. [8] identified that: 1) peptide AIP1–7 showed homology with *Hp* plasminogen-binding protein (PBP) and with the enzyme ubiquitin-protein ligase E3 component n-recognin 2, expressed in pancreatic acinar cells; and 2) the antibody against PBP was associated with AIP. *Hp* was found to bind plasminogen by *Hp* PBPs, enhancing its virulence [9]. These data further support our proposition that *Hp* might trigger AIP through molecular mimicry and its increased virulence, plasminogen binding and its conversion to plasmin is the only proteolytic activity of *Hp* may enhance tissue damage [9] and is involved in carcinogenesis. Therefore, *Hp* eradication may positively influence patients with autoimmune disorders including AIP by ameliorating the autoimmune sequelae [10].

Conflict of interest statement

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Letter: is *Helicobacter pylori* behind Barrett's oesophagus and colorectal neoplasms?

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Sirs, Andrichi *et al.'s* meta-analysis[1] showed that Barrett's oesophagus (BO) was associated with an increased risk of both colorectal adenomas and colorectal cancer (CRC). They concluded that if the risk estimates for CRC in BO patients reflects a real relationship, an established association will warrant a search for common genetic or environmental risk factors.

Some genetic alterations are common in both conditions[2]; we initially found that specialised intestinal metaplasia indicating BO appeared in a significant percentage of patients with colon tumours (12/23) compared with controls (2/14) and was associated with increased oesophageal mucosal expression of oncogenes Ki-67 and p53/Bcl-2[3] that indicated mainly increased proliferation leading to oncogenesis.

In this regard, *Helicobacter pylori* infection may be considered a promoter of both diseases. Our and others' data[4] indicate that *H. pylori* infection might contribute to oesophageal adenocarcinoma progress in subpopulations with gastro-oesophageal reflux disease and BO. In this respect, gastrin, induced by *H. pylori* infection, is an oncogenic growth factor contributing to oesophageal, gastric and colon carcinogenesis and, in particular, playing a potential causal effect on neoplastic progression in BO and left side CRC showing, for instance, anti-apoptotic activity through upregulation of the anti-apoptotic Bcl-2 and stimulation of mutagenic and tumourigenic cyclooxygenase-2 expression.[4–6]

Moreover, *H. pylori* infection is mostly frequent in colonic adenomas and tumour tissues (documented by immunohistochemical stain) and is accompanied by increased cell proliferation (mainly enhanced Ki-67 and Bcl-2 expression) and impaired apoptotic (decreased Bax) processes, thereby indicating its potential pathogenetic role.[6–8]

Apart from upper gastrointestinal tract (UGT), *H. pylori* infection might also cause chronic inflammatory colon mucosal damage and stimulate cancer stem cells and/or recruit bone-marrow–derived stem cells, which may ultimately facilitate UGT and colon tumour formation and progression.[8–10] However, further studies are needed to elucidate the proposed pathophysiological mechanisms involved in *H. pylori*-associated colon oncogenesis; its eradication might inhibit these oncogenic processes.

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Letters to the Editors

Letter: is *Helicobacter pylori* behind Barrett's oesophagus and colorectal neoplasms? Authors' reply

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Sirs, We thank Dr Kountouras and colleagues for their interest in our article.[1, 2] The role of *Helicobacter pylori* in the development of peptic ulcers, as well as gastric adenocarcinomas and gastric mucosa-associated lymphomas (MALT lymphomas), is now widely accepted.[3] The idea that the same pathogen could be involved in the pathogenesis of both Barrett's oesophagus (BO) and colonic tumours is intriguing.

The recent findings by Zhang *et al.*[4] showing an adjusted odds ratio of 1.18 (95% CI: 1.01–1.38) between *H. pylori* infection and colorectal cancer points to a potential association between the two. Furthermore, a meta-analysis[5] of 13 studies from 2008 also showed a positive association, with an odds ratio of 1.49 (95% CI: 1.17–1.91). However, studies by Abbass *et al.*[6] (published after the meta-analysis by Zhao *et al.*[3] and therefore not included in the analysis) and Siddheshwar *et al.*[7] found no association.

Given the uncertainty at present, more studies are needed to confirm the relationship, should one exist, as well as a mechanistic explanation.

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Letter: is blood transfusion really a risk factor for rebleeding in nonvariceal gastrointestinal bleeding?

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Sirs, I read the article by Restellini *et al.* with interest.[1] The authors analysed 1677 patients with nonvariceal upper gastrointestinal bleeding, collecting clinical parameters (gender, age, comorbidity, ASA score, melaena, haematemesis, drug usage, etc.) and correlated these with rebleeding. After adjusting for confounders in multivariable analysis, they found transfusion of red blood cells within 24 h of presentation to be significantly and independently associated with an increased risk of rebleeding (OR: 1.8, 95% CI: 1.2–2.8, $P = 0.005$).

I am afraid that there are some points which need to be discussed to avoid misleading readers. Restellini *et al.'s* finding may be new, because using blood transfusion as a predictor for rebleeding has not been mentioned in recent consensus, ACG guidelines or review articles.[2–4] From a clinical point-of-view, doctors usually order blood transfusion for patients with low haemoglobin or unstable vital signs after resuscitation.

Recent ACG guidelines suggest a blood transfusion if haemoglobin is below 7 g/dL.[3] In Restellini *et al.'s* observation, patients who received a blood transfusion may indicate a severe condition in this group therefore the rebleeding rate is theoretically high. It is unreasonable to regard blood transfusion as the cause of rebleeding.

There are some frequently used scoring systems for predicting prognosis which can be used to inspect this

NISIN and *Clostridium difficile*: A Potentially Effective Treatment for an Increasingly Problematic Disease

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To the Editor: The paradigm of *Clostridium difficile*-associated diarrhea (CDAD) is changing. Its incidence and severity are increasing. The emergence of hypervirulent strains of *C. difficile* is worrisome. Treatment failure rates are on the rise, and recurrence rates in treated patients are disappointing. Lately, CDAD has been described in populations previously thought to be at low risk.

Nisin is a peptide antibiotic that exhibits bactericidal activity against Gram-positive organisms. It belongs to a class called the lantibiotics and is produced by the bacterium *Lactococcus lactis* subsp. *Lactis* (1). Nisin is safe and was granted GRAS status (Generally Recognized As Safe; notice no. GRN 000065) by the United States Food and Drug Administration. It is currently used as an additive to prevent food spoilage.

The mode of action of nisin has been studied extensively. The cytoplasmic membrane is the primary target of nisin. It incorporates into the cytoplasmic membrane, leading to the formation of transient multistate pores that allow the efflux of low-molecular mass molecules such as amino acids, K^+, and internal adenosine triphosphate. As a result, both the membrane potential and pH gradients across the cytoplasmic membrane are dissipated, leading to cell lysis and death (2). More recently, nisin was found to interact with the lipid II molecule, which is an essential membrane-bound precursor for cell-wall biosynthesis (3).

Nisin exhibits excellent *in vitro* activity against *C. difficile*. In fact, Bartoloni *et al.* (4) tested metronidazole, vancomycin, and nisin against 60 toxigenic strains of *C. difficile* collected from subjects with CDAD in hospital settings and found nisin to be eight times more effective than the current standard treatments (4). Preliminary animal studies have shown that nisin is poorly absorbed from the large intestine, which makes systemic toxicity less likely. It also attains high concentrations in the intestinal lumen and has no toxic effects on the intestinal epithelium (5). Nisin's other advantage is its poor bactericidal activity against the most common gastrointestinal microbiota, *Bacteroides* and *Prevotella* (4). Nisin undergoes proteolytic degradation in the upper gastrointestinal tract, hence, it will require delivery directly to the colon of infected patients. Options include administering the medication as an enema or perhaps oral delivery via a coated capsule that will release the intact drug at the target site. For the reasons mentioned, nisin appears to be an excellent candidate agent for the treatment of CDAD and it merits further investigation in phase I clinical trials.

CONFLICT OF INTEREST

Guarantor of the article: Ahmad H. Ali, MBBS.
Specific author contributions: Ahmad Ali is the principal writer of the manuscript with inspiration and direction of Thomas Hale. Roger Smalligan is the co-writer of the manuscript. Robert Urban, Faisal Khasawneh and Harold Werner participated in editing the paper.
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Impact of *Helicobacter pylori* Infection on Colon Oncogenesis

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To the Editor: Sonnenberg and Genta's large-scale series (1) showed that various forms of gastritis related to *Helicobacter pylori* infection (*Hp*-I) confer an increased risk for colonic neoplasms; *Hp* organisms were demonstrated by a polyclonal anti-*Hp* immunohistochemical stain, ensuring the highest possible histopathological detection rate. The authors concluded that the pathophysiological mechanisms underlying the association between *Hp* and colonic neoplasms are not fully clear, although *Hp*-related gastrin, as an oncogenic factor, might play a role in colon oncogenesis and/or *Hp* could act on the colon mucosa itself and affect neoplasm growth (1).

Based on histology, our preliminary data indicated *Hp* presence in malignant tissue in 34 of 41 (82.9%) patients with colorectal cancer (CRC) (23 men, mean age 73.6±7.9 years) (2). Extending these data to 50 patients with CRC, 25 patients with colorectal adenomas, and 10 controls, we found a significantly higher presence of *Hp*-I in the adenoma (68%) and CRC (84%) groups compared with controls (30%), confirming our preliminary data (3). It is important to note that *Hp* presence was documented by immunohistochemical stain (using polyclonal rabbit anti-*Hp* antibody) in adenoma and malignant colonic tissues, also used by the authors (1). The presence of *Hp*-I with accompanying

immunohistochemical expression of CD44 (an indicator of cancer stem cells (CSCs) and/or bone marrow-derived stem cells (BMDSCs)) in biopsy specimens was found in a high proportion of patients with colorectal adenomas accompanied with moderate/severe dysplasia (88%) and CRC patients with moderate/severe degree of malignancy (91%) (3,4). Comparable pictures were also obtained for oncogene Ki67, anti-apoptotic Bcl-2 protein, and CD45 (an indicator of immune surveillance locally) immunohistochemical expressions (3).

Therefore, casting further light in the not entirely clear pathophysiological mechanisms underlying the association between *Hp* and colonic neoplasms, apart from gastrin mentioned by the authors (1), our own results indicate that *Hp*-I has an impact on colon and its oncogenesis by: causing a possible chronic inflammatory mucosal damage, comparable to the upper gastrointestinal tract (UGT) inflammation; stimulating CSCs or recruiting BMDSCs, similar to UGT *Hp*-I-associated chronic inflammation, hyperplasia, metaplasia, dysplasia, and BMDSC engulfment that may facilitate tumor formation and progression in animal models and humans (5); and affecting oncogenes and immune surveillance processes that may be involved in the sequence: colon epithelium transformation to adenoma–moderate to severe dysplasia–CRC development/progression. Thus, *Hp* eradication might inhibit the development or delay the progression of CRC. However, large-scale future studies are warranted to elucidate the proposed pathophysiological mechanisms involved in *Hp*-associated colon oncogenesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Appendectomy Is Not Associated With Adverse Outcomes in *Clostridium difficile* Infection: A Population-Based Study

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To the Editor: The appendix harbors commensal microbes, may have immunoregulatory functions and may serve as a microbial reservoir and facilitate repopulation of colonic microbiota disrupted by antibiotic use (1). Alteration of normal intestinal microbiota, usually in the setting of antibiotic use, allows *Clostridium difficile* to proliferate and cause disease (2). Bacterial biofilms within the mucus that lines the appendiceal epithelium may facilitate reinoculation of the gut microbiome after such disruption (1). There is conflicting evidence that appendectomy increases the risk of recurrent *C. difficile* infection (CDI) (3,4), and a study has shown that appendectomy decreases the risk of primary CDI (5). If the appendix serves as a bacterial reservoir and supports the repopulation of normal colonic flora, then it is plausible that appendectomy would increase the risk of adverse outcomes from CDI. We assessed outcomes (severe CDI, severe-complicated CDI, treatment failure, and recurrence) in a population-based cohort of adults with and without prior appendectomy.

We included adults (≥18 years) from a population-based study of CDI in Olmsted County from 1991 to 2005 (6). Severe CDI was defined by a peripheral leukocyte count ≥15,000/μl or ≥50% serum creatinine rise from baseline; severe-complicated CDI was defined by megacolon, sepsis, or death due to CDI (6,7). Recurrence was defined by recurrent diarrhea and a positive stool assay for CDI within 8 weeks of initial diagnosis after symptom resolution. Treatment failure was defined as a change in treatment within 14 days due to nonresponse (6,7). Appendectomy status was determined by chart review using the Rochester Epidemiology Project.

There were 355 CDI cases and 46 (12.9%) had undergone appendectomy before CDI. The latter group was younger (median age 64 vs. 71 years, $P=0.08$), and more likely female (odds ratio (OR) 2.9, 95% confidence interval (CI) 1.3–6.9, $P<0.01$), but had a similar Charlson Comorbidity Index (CCI) compared with patients without appendectomy. The median time from appendectomy to CDI was 22.5 years (range 5 days–45 years). There were no differences in the frequency of prior antibiotic exposure (OR 1.6, 95% CI 0.5–4.7, $P=0.36$) or gastric acid suppression (OR 1.2, 95% CI 0.6–2.2, $P=0.59$) between the groups. On univariate analyses, there were no differences in treatment failure (OR 0.9, 95% CI 0.4–1.9, $P=0.77$) or the development of severe (OR 0.6, 95% CI 0.3–1.4, $P=0.26$), severe-complicated (OR 0.8, 95% CI 0.2–2.8, $P=0.76$), or recurrent CDI (OR 0.9, 95% CI 0.5–1.9, $P=0.93$) in those with as compared with those without prior appendectomy. On multivariate logistic regression analysis, adjusting for age, sex, and comorbidities, appendectomy was not associated with adverse CDI outcomes. However, a higher CCI was associated with severe and severe-complicated CDI, and increasing age was associated with severe-complicated CDI.

In summary, patients who had undergone appendectomy before the development of CDI had no differences in CDI risk factors, treatment, or outcomes including treatment failure, development of severe or severe-complicated CDI,

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LETTER TO THE EDITOR

Helicobacter pylori Infection and Serum Adiponectin

To the Editor,

We read with interest the paper by Ando et al. [1] who reported that *Helicobacter pylori* (*Hp*) eradication treatment resulted in elevation of serum total adiponectin and its isoforms levels in an adult Japanese population. More specifically, total, high- (HMW), middle- (MMW), and low-molecular weight (LMW) adiponectin levels were similar between *Hp* immunoglobulin G (IgG) antibodies positive (n = 338) and negative (n = 118) individuals who underwent upper gastrointestinal endoscopy for dyspepsia. Furthermore, total and HMW adiponectin increased after successful (n = 241) or unsuccessful (n = 97) *Hp* eradication regimen, whereas MMW and LMW adiponectin increased only after successful treatment. These findings add to the existing evidence and warrants further investigation.

It seems paradoxical why adiponectin increases, despite the increase in body mass index and abdominal circumference, regarded as a sufficient index of abdominal adiposity, given that adiponectin is the only adipocytokine which decreases as abdominal adiposity increases [2]. The speculation that the *Hp*-induced decrease in tumor necrosis factor (TNF)-α is partly responsible may not be sufficient, because: (1) the adiponectin increase cannot be explained in the group with unsuccessful *Hp* eradication, in which TNF-α levels are not expected to be significantly modified, (2) post-treatment systemic inflammation was not changed in either group, at least as estimated by high-sensitivity C-reactive protein, and (3) we have previously published a positive correlation between *Hp* IgG antibody titer and not only TNF-α (r_s = 0.426, p = .006), but also total (r_s = 0.415, p = .002) or HMW (r_s = 0.412, p = .002) adiponectin in patients with nonalcoholic fatty liver disease (NAFLD) [3]. Likewise, statistically significant higher levels of both total and HWM adiponectin were observed in ^{13}C urea breath test positive than negative patients (total adiponectin (mean ± standard error of the mean): 5.0 ± 0.8 versus 3.9 ± 0.5 μg/mL, respectively, p = .048; HMW adiponectin: 2.8 ± 0.4 versus 1.9 ± 0.3 μg/mL, respectively, p = .036) [3]. Although we have also no sufficient explanation and these results were opposite to our initial hypothesis, we had speculated that *Hp* infection might trigger induction of TNF-α, whereas adiponectin is secondarily increased to counterbalance the pro-inflammatory cascade. In this setting and given that *Hp* IgG antibodies were measured by the authors [1], it would be of particular interest, if they performed a post hoc correlation analysis between *Hp* IgG antibody and total adiponectin or its isoforms.

Given the similar effect of *Hp* eradication treatment in either group, at least on total and HMW adiponectin levels, an *Hp*-independent contributor might be suspected; in this setting, gut microbiota modulation render an appealing speculation, as the authors reported [1]. Indeed, the 2-week combination of norfloxacin and ampicillin was shown to increase serum total adiponectin in *ob/ob* obese and insulin-resistant mice without affecting adiposity [4]; however, simultaneous decrease in glucose tolerance and corresponding serum insulin levels during the oral glucose tolerance test was also significantly lower in antibiotic-treated than in control or pair-fed mice, despite similar food intake and body weight among groups. Furthermore, the antibiotic-treated mice showed reduced liver, but not serum, triglycerides levels, and hepatic steatosis, indicating improvement in hepatic insulin resistance (IR). This may, at least partly, occur through increased expression of Cyp4A10, a marker for microsomal fatty acid oxidation, and decreased expression of certain lipogenic genes triggered by antibiotic treatment [4]. Serum lipopolysaccharides (endotoxins from gut gram-negative bacteria playing an important role in the development of IR) and TNF-α were also decreased in antibiotic-treated mice [4]. In view of all aforementioned data, antibiotic treatment seems to decrease both hepatic and systematic IR without affecting body weight or adiposity. According to this speculation, adiponectin increase observed by the authors (1) may represent a consequence of decrease in IR in both groups rather a direct effect of antibiotic treatment. Because serum glucose levels were measured [1], it would be of particular interest, if the authors had frozen serum samples and performed a post hoc measurement of insulin levels to estimate IR (i.e., by homeostatic model of assessment IR) between (1) *Hp*-positive and *Hp*-negative patients, and (2) those with successful or unsuccessful *Hp* eradication treatment; IR could be different, despite the similar glucose levels found between groups. Because the only two nonrandomized open-label controlled

trials investigating the effect of *Hp* eradication on IR [5] provided controversial results, this would be probably the largest and best designed study evaluating this issue, even though post hoc.

Finally, the effect of proton pump inhibitors, used in *Hp*-positive patients together with combined antibiotic treatment, on serum adiponectin levels cannot be excluded; however, there are currently no relevant experimental or human data.

In conclusion, an increase in serum adiponectin levels after short-term antibiotic treatment would be of importance, although this requires validation by other studies assessing its long-term effect. If confirmed, antibiotic treatment, although still unclear by which mechanisms, might have clinical implications on IR and other related morbidities, including type 2 diabetes mellitus and NAFLD.

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Is *Helicobacter pylori* the usual suspect behind gastroesophageal reflux disease and dacryostenosis?

Dear Editor,

The hypothesis by Magliulo et al. [1] proposes that the ascending products (i.e., pepsin) of gastroesophageal reflux (GER) could cause edema of the nasolacrimal duct mucosa, which might progress to fibrosis and chronic inflammation and, ultimately, to dacryostenosis (DS); DS may be affected by GER disease (GERD) by the direct noxious effect of the refluxate on the nasal cavity surrounding the nasolacrimal duct, thereby inducing an ascending inflammation to the duct [2].

Relative data indicate that: descending inflammation from the eye or ascending inflammation from the nose contributes to DS development; dacryocystitis associated with DS is caused by bacteria; trefoil factor family (TFF) peptides regulate the human lacrimal system, and their deficiency may contribute to DS development; and other antimicrobial peptides (defensins) have a therapeutic potential in dacryocystitis, i.e., they have a broad spectrum of antimicrobial activity and accelerate epithelial healing. However, caution is warranted as defensins also promote fibrin formation and cell proliferation, key elements in DS; in particular, human-beta defensin (HBD)-2 is detected only in the lacrimal passage in the presence of bacterial dacryocystitis [3].

Our relative data indicate that: *Helicobacter pylori* infection (*Hp*-I) is possibly involved in GERD pathophysiology [4]; and *Hp* bacteria exist in ophthalmic tissue, causing ophthalmic pathologies [5], possibly including DS, by releasing various proinflammatory cytokines, thereby contributing to the mentioned descending eye inflammatory process. Moreover, *Hp* has been found in the nasal and oral cavities, the oral cavity might act as a permanent reservoir and *Hp* may reach the eye through the nasal cavity [5], possibly causing DS by the mentioned nose ascending inflammation. Moreover, *Hp* markedly upregulates HBD-2 expression, a proinflammatory mediator involved in *Hp*-related inflammatory pathologies and reduces TFF protein expression causing damage, thereby further contributing to DS pathophysiology.

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Letter to the Editor

Aquaporin 4, *Helicobacter pylori* and potential implications for neuromyelitis optica ☆

Keywords:
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Dear Editor,

We have read with interest the paper by Vaishnav et al. (2013), who investigated the hypothesis that antibodies to aquaporin 4 (AQP4) associated with neuromyelitis optica (NMO) can be triggered by exposure to environmental plant and bacterial proteins; high similarity to a known epitope for NMO-IgG, AQP4(207–232), was observed for corn ZmTIP4-1, and NMO patient serum showed reactivity to both peptides and to plant tissue, findings warranting further investigation regarding the role of the environmental agents in NMO etiology. Discussing their results, the authors considered that molecular mimicry is a mechanism by which exogenous agents, including plant, bacterial and viral proteins, may trigger immune responses against self or non-self antigens.

Apart from plant protein(s), reported by the authors (Vaishnav et al., 2013), *Helicobacter pylori* (*Hp*), a gram-negative bacterium that affects more than 50% of the world's population, might be a common pathogen triggering different neurological disorders of the central nervous system (CNS) including multiple sclerosis (MS)/NMO, cerebrovascular diseases, mild cognitive impairment, Alzheimer's disease, Parkinson's disease, seizure disorders, migraine and ophthalmic disorders, namely glaucoma (defined as "ocular AD"), and anterior optic ischemic neuropathy (Kountouras et al., 2006; Gavalas et al., 2007; Kountouras et al., 2010; Deretzi et al., 2011). Current *Hp* infection (*Hp*-I) induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves, thereby contributing and possibly perpetuating neural tissue damage (Kountouras et al., 2006, 2007, 2008a). Moreover, eradicating *Hp*-I may alter MS/NMO pathophysiology.

In this respect, Li et al. (2009) found that *Hp* neutrophil-activating protein (*Hp*-NAP) may be associated with the anti-AQP4 antibody-related neural damage in multiple sclerosis (MS)/NMO patients. However, this conclusion might be incomplete, because *Hp*-NAP did not bind to anti-AQP4 antibody, and thus molecular cross mimicry between *Hp*-NAP and AQP4 is unlikely to be a responsible underlying mechanism. On the other hand, the authors claimed that *Hp*-NAP by promoting neutrophil and monocyte recruitment/activation and inducing mast cells to release proinflammatory molecules able to activate these cells, could contribute to neural oxidative damage in MS/NMO patients. However, although the authors showed higher *Hp* seropositivity rates in anti-AQP4 antibody-positive MS/NMO (AQP4+/MS) patients, they disclosed a significantly lower *Hp* seropositivity rate in conventional MS patients than in healthy controls and a significant inverse association with mean Expanded Disability Status Scale score and fulfillment of the Barkhof criteria for brain MRI lesions; they concluded that *Hp*-I is a potential protective factor against conventional MS in Japanese (Li et al., 2007), which contradicts their latest conclusion that *Hp*-I seems to be one of the risk factors for the development of AQP4+/MS (Li et al., 2009). Finally, the authors mentioned that a similar low prevalence of *Hp*-I was also reported in the Western population (Wender, 2003), which is again incomplete.

The variable role played by *Hp* in the pathogenicity of MS may be predicated on ethnicity (Kountouras et al., 2008a,b). In this respect, by using histology, recognized as the practical gold standard for the diagnosis of current *Hp*-I, our series showed a strong association between *Hp*-I and MS Caucasian patients (Gavalas et al., 2007). Extending our own findings, we also observed a strong association between *Hp*-I and clinically isolated syndrome (CIS) that describes a first clinical episode with features suggestive of MS, again in a Greek cohort [*Hp*-I presence by histology in 89.6% of patients and in 50% of controls; odds ratio 8.6 (95% CI 2.4–30.78), $p < 0.001$]. Moreover, *Hp*-I might play a critical role in the pathogenesis of both systemic sclerosis (SSc) and MS and additionally the coexistence of SSc and MS even in the same patient has been described, thereby suggesting that *Hp*-I might be a common underlying factor predisposing for the development of both diseases (Kountouras et al., 2011). It was recently shown that microbial peptides with limited sequence homology to myelin basic protein can induce MS-like disease in mice humanized for a T-cell receptor derived from an MS patient (Harkiolaki et al., 2009). *Hp*-I seems to be one of the risk factors for the development of anti-AQP4+/MS. Genetic factors and *Hp*-I are risk factors only for anti-AQP4 antibody positive NMO/NMO spectrum disorder (NMOSD) but not for anti-AQP4 antibody negative NMO/NMOSD (Kountouras et al., in press; Yoshimura, 2013).

In this regard, a series of *Hp*-related factors have been implicated in inducing blood–brain barrier (BBB) disruption, including inflammatory mediators (e.g., cytokines, chemokines induced by *Hp*-I) and oxidative stress (Kountouras, 2009). Consequently, the *Hp*-induced BBB disruption might permit access of AQP4-specific antibodies and T lymphocytes to the CNS, thereby playing a major role in NMO pathogenesis (Li et al., 2009; Kountouras et al., 2010, in press). In addition, *Hp*-NAP, as a virulence factor, recruits leukocytes from the vascular lumen, and activates neutrophils, monocytes and mast cells involved in BBB disruption (Kountouras, 2009; Kountouras et al., in press). Moreover, *Hp*-induced VacA cytotoxin promotes intracellular survival of the bacterium, modulates host immune responses and induces autophagy (Kountouras et al., in press). Subsequently, *Hp* as an intracellular microorganism invades and replicates in the cells. The autophagy induction by *Hp* is not only found in macrophages, but also in dendritic cells and gastric epithelial cells. The bacterium's residence inside infected cells will increase its resistance to antimicrobial treatment, avoid neutralization by anti-*Hp* antibodies, impair antigen presentation, and alter the cellular immune response. In turn, the potential influx of activated monocytes infected with *Hp* through the disrupted BBB in the brain might lead to microglia activation and to degeneration in the brain partially by potential

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activation of natural killer cells and interferon-γ production, detrimental for MS (Kountouras et al., in press). Therefore, apart from *Hp*-NAP, *Hp*-I itself, by inducing several mediators and/or sharing some potential homologous epitopes (molecular mimicry) with neural components might be a common denominator influencing the pathophysiology of MS/NMO (including relapsing type) (Kountouras et al., 2012). However, further studies are needed to elucidate the role of the environmental agents including *Hp* in NMO etiology.

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