Factors modulating the outcome of treatment for the eradication of *Helicobacter pylori* infection

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**SUMMARY**

A group of 180 *H. pylori* culture positive dyspeptic patients (64 patients with peptic ulcer, PU) completed a 2-week treatment with omeprazole, amoxicillin and metronidazole and underwent endoscopy again 6-8 weeks after the end of therapy. One hundred and twenty-four patients (68.8%) were successfully treated. Factors increasing the rates of eradication were the presence of PU (*p* = 0.007) and anti-CagA serum antibodies (*p* = 0.003). Factors negatively modulating eradication were the presence of coccoid forms (*p* = 0.0008) and metronidazole-resistant strains (*p* = 0.001); degrees of histological gastritis had no significant effect on eradication rates. Microscopic examination of smeared biopsies for the detection of the coccoid morphotype of *H. pylori* may help avoiding therapeutic failures.

Key words: *H. pylori* infection, CagA, Coccoid forms, Peptic ulcer, Eradication treatment.

*Helicobacter pylori* infection is the main cause of chronic gastritis, the most frequent chronic bacterial disease after tooth decay, and the main etiologic factor in the development of peptic ulcer disease and malignant gastric lesions (Suerbam & Michetti, 2002). The treatment regimens of the infection include the administration of two or three antibiotics and a proton pump inhibitor, with or without bismuth salts, for one or two weeks (Rimbara *et al.*, 2011). Therapeutic success depends on many factors, the most important being chemoresistance (Vakil & Mégraud, 2007). Most studies in the literature have considered only a few of the numerous determinants of reduced efficacy (Sugimoto & Yamaoka, 2009; Yakoob *et al.*, 2011). The present study investigated the possible influence of some factors upon the eradication of *H. pylori* infection.

The experimental design was to enrol for the treatment of *H. pylori* infection all patients with peptic ulceration (PU, at least 50 patients) occurring in the period of one year in our department and a group of patients with non-ulcer dyspepsia (NUD), randomly chosen (at least 100 patients). All patients underwent upper endoscopy for dyspepsia and gave their written informed consent. The local Ethical Committee gave its approval. No patient had previously received antibiotics commonly used in anti-*H. pylori* therapy or proton pump inhibitors (PPI) in the previous three months. Twelve biopsies were taken per patient, four biopsies for each site, antrum, corpus and fundus, using sterile forceps. Forceps were disinfected and rinsed under running water between biopsies and were changed from one site to the other. Biopsies were used for rapid urease test, microscopic examination of smeared mucosa samples after staining with acridine orange, histology and culture on selective agar plates (that contained 10% foetal bovine serum, trimethoprim 10 mg/L, vancomycin 10 mg/L, cefsulodin 5...
mg/L and amphotericin B 5 mg/L) and on selective blood agar with incorporated 8 mg/L of metronidazole. Plates were incubated in microaerobic atmosphere for ten days and examined daily for suspected colonies starting from the fourth day. Patients were also tested for the presence of anti-CagA serum antibodies, using a household Western blotting technique. Briefly, the proteins of *H. pylori* type strain CCUG 17874 were denatured and resolved electrophoretically in acrylamide-bisacrylamide gel, transferred electrophoretically onto a nitrocellulose sheet and let react with the patients’ serum samples (diluted 1:100) overnight at room temperature. The immunoreaction was detected by using a goat anti-human IgG serum conjugated with peroxidase and revealed by the addition of substrate. A rabbit serum raised against purified CagA (gift of R. Rappuoli, Novartis, Siena) was used as control. Histological sections were stained with haematoxylin-eosin and modified Giemsa stain for bacterioscopic examination and results were given according to the Updated Sydney System (Dixon*et al.*, 1996). Suspected colonies on plates were examined by Gram stain and oxidase, catalase and urease tests. Three to five colonies per each site grown on primary plates were subcultured onto blood-agar plates containing 8 mg/L of metronidazole. If at least one colony grew, the clone was considered resistant. *H. pylori* organisms growing on primary selective medium supplemented with metronidazole 8 mg/L were considered resistant, too. Resistance to amoxicillin was not investigated.

The total number of dyspeptic patients examined initially was 271; 200 patients were infected by *H. pylori* and the culture of at least one biopsy gave positive results in 185 cases: 64 cases with PU and 121 cases with NUD. All the 185 culture positive patients were treated for two weeks with omeprazole 20 mg *b.i.d.*, amoxicillin 1000 mg *b.i.d.* and metronidazole 500 mg *t.i.d.*. A total of 182 patients completed the treatment: 64 patients with PU and 118 with NUD. Six weeks *ca.* after the end of therapy, patients underwent endoscopy again and biopsies were processed as above reported. Patients were considered still infected if culture or at least two of the other tests disclosed *H. pylori* organisms.

Data were analysed using the chi-square test with the Mantel–Haenszel correction or 1-tailed Fisher exact test when necessary; *p* <0.05 was considered statistically significant.

A total of 180 patients repeated endoscopy; two patients with NUD refused the examination. The mean (SD) age of patients was 57.42 (8.59) years, range 27-87 years. Twelve patients out of 64 patients with PU had gastric ulcer and 52 patients had duodenal ulcer (Table 1). Out of 116 patients with NUD, 60 patients had macroscopically normal gastroduodenal mucosa and 56 patients showed an inflamed mucosa (Table 1).

Histology in NUD cases gave the following results: normal mucosa in at least one site, corpus or fundus, was found in 28 cases; the results reported in the other 88 cases are the average of the histological lesions observed in the three gastric areas: mild/moderate, non-active chronic gastritis (CG) was observed in 50 cases; severe, active CG was found in 38 cases (Table 1). The presence of atrophic lesions was very rare and therefore was ignored.

Coccoid forms of *H. pylori* were observed in six cases (3.3%) (Fig. 1B).

Metronidazole-resistant strains were isolated in 12 cases (6.6%) by examining 3-5 colonies developed on primary plates and in 52 cases (28.8%) by streaking biopsies onto selective agar containing metronidazole at the threshold concentration of 8 mg/L (*p* <0.0001) (Table 1).

Anti-CagA serum antibodies were detected in 135 patients (75.0%), 58 patients of whom had PU (90.6%) and 77 patients had NUD (66.3%), (*p* =0.0003, OR = 4.9, CL 1.83-13.85) (Table 1). A total of 124 patients (68.8%) were successfully treated; the factors that increased the rates of the eradication treatment were the presence of PU vs. NUD (*p* =0.007, OR=2.65, CL 1.21 to 5.89), the presence of anti-CagA serum antibodies (*p* = 0.003, OR 2.84, CL 1.33-6.09), the absence of coccoid forms (*p* =0.0008, OR undefined) and the absence of metronidazole-resistant strains (*p* =0.001, OR 2.9, CL 1.39-6.04). In the presence of histological inflammation in all gastric areas, the percentage of bacteriological healing was increased (67.0 vs. 57.1 in cases in which histological normal mucosa was observed in either corpus or fundus gastric areas) but the difference did not reach statistical significance (*p* =0.341, OR=1.53). In cases of severe, active CG, 57.8% of patients were treated successfully vs. 74.0% of patients with...
mild/moderate, non-active CG ($p=0.113$, OR=2.07, non-significant).

Triple therapy for at least one week is sufficiently effective in most patients, even though recent protocols, such as the so-called sequential therapy, seem more successful (Gatta et al., 2009). The mechanisms that may promote increased eradication rates of H. pylori infection are still speculative. Many studies on this subject have been published, but works considering all the principal

### TABLE 1 - Factors that might modulate the eradication of H. pylori infection.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of patients treated</th>
<th>No. and percentage (%) of patients cured</th>
</tr>
</thead>
<tbody>
<tr>
<td>PU</td>
<td>64</td>
<td>58 (81.2)</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
<td>52</td>
<td>40 (76.9)</td>
</tr>
<tr>
<td>Gastric ulcer</td>
<td>12</td>
<td>12 (100)</td>
</tr>
<tr>
<td>NUD</td>
<td>116</td>
<td>72 (62.0)</td>
</tr>
<tr>
<td>Macroscopically normal mucosa</td>
<td>60</td>
<td>36 (64.2)</td>
</tr>
<tr>
<td>Macroscopically inflamed mucosa</td>
<td>56</td>
<td>36 (64.2)</td>
</tr>
<tr>
<td>Histologically normal mucosa in one site</td>
<td>28</td>
<td>16 (57.1)</td>
</tr>
<tr>
<td>Mild/moderate, non-active chronic gastritis</td>
<td>50</td>
<td>37 (74.0)</td>
</tr>
<tr>
<td>Severe, active chronic gastritis</td>
<td>38</td>
<td>22 (57.8)</td>
</tr>
<tr>
<td>Presence of coccoid forms</td>
<td>6</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Absence of coccoid forms</td>
<td>177</td>
<td>124 (70.0)</td>
</tr>
<tr>
<td>Presence of anti-CagA serum antibodies</td>
<td>135</td>
<td>101 (74.8)</td>
</tr>
<tr>
<td>Absence of anti-CagA serum antibodies</td>
<td>45</td>
<td>23 (51.1)</td>
</tr>
<tr>
<td>Metronidazole-resistant organisms</td>
<td>52</td>
<td>27 (51.9)</td>
</tr>
<tr>
<td>Metronidazole susceptible organisms</td>
<td>128</td>
<td>97 (75.7)</td>
</tr>
</tbody>
</table>

FIGURE 1 - Gastric biopsies smeared on glass slides and stained with acridine orange. A, normal spiral H. pylori organisms. B, coccoid forms (yellow arrows) and scanty spiral (white arrows) H. pylori organisms.
determinants of reduced efficacy are very few. The present study evaluated the presence of PU and macroscopic gastric mucosa alteration (redness, erosions etc.), the degree of histological mucosal inflammation and genotype, chemosusceptibility and the presence of coccoid forms of the infecting organisms. Factors that increased eradication rates significantly were the presence of PU vs. NUD, seropositivity for CagA and the absence of H. pylori organisms with coccoid morphology, in addition to bacteria with normal spiral shape, in at least one biopsy (Table 1 and figure 1). It is debated whether patients with PU or those infected by the CagA-positive (CagA+) genotype can resolve the infection more easily (Broutet et al., 2003; Sugimoto & Yamaoka, 2009; Zhao et al. 2007; Huang et al., 2005). The increased frequency of therapeutic success in PU patients may be due to the local improved diffusion of antibiotics and their enhanced penetration from the blood stream. These phenomena may occur for different reasons: the presence of ulcer, the degradation of the mucous layer (which commonly takes place around mucosal ulceration) and the high degree of epithelial inflammation surrounding the lesions, which could facilitate the delivery of antibiotics by altering the vascular and epithelial permeability.

In the literature, there are 17 studies on CagA (or cagA) status and the success of H. pylori infection treatment. Sugimoto et al. (2009) revised the dedicated literature and found that in only half of the 16 studies they examined, eradication was achieved in a significantly greater number of cases in patients infected by cagA+ strains. However, combining the cumulative data, they observed that the patients treated successfully were more likely to be cagA+ than cagA-: 83.1% vs. 69.9%, respectively (p<0.01). There may be many reasons for straightforward healing: 1. Patients infected by CagA+ organisms develop PU more frequently (at least, in this area; NF personal observation) (Tovey et al., 2006). 2. cagA+ strains are reported to grow faster in vitro (Censini et al., 1996); since antibiotics interfering with the metabolism of dividing cells, such as amoxicillin, are more efficacious during bacterial cell division, cagA+ organisms would be more susceptible and be destroyed more rapidly than the cagA- ones, which may be in resting phase. 3. Strains expressing CagA enhance the mucosal production of proinflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, which are potent inhibitors of the acid secretion (Wang et al. 1999). Increased cytokine levels in the gastric mucosa colonized by CagA+ organisms could therefore result in a strong acid suppression (Furuta et al. 2002) and therefore in an enhanced efficacy of the treatment (Grayson et al., 1989; Sugimoto et al., 2007).

High degrees of chronic inflammation of corpus gastritis may significantly increase the eradication rates (Georgopoulos et al., 2000). Nonetheless, our results differ and are partially contradictory: while the absence of histological inflammation in the gastric corpus or fundus reduced the rates of eradication, the presence of a mild, non-active CG increased the frequency of therapeutic success with respect to the cure rates in cases of severe active CG; in both cases, however, the differences were not statistically significant. This subject is still debated.

To the best of our knowledge, treatment failure in individuals harbouring coccoid H. pylori organisms has been described for the first time in this study. In natural conditions, H. pylori organisms are able to resist antibiotic treatment in two principal ways: through biofilm formation (Makipura & Friedenberg, 2011), which may prevent the drug from diffusing properly, and by reverting to a viable but non-cultivable state and remaining in a coccoid morphotype (Andersen & Rasmussen, 2009). On the basis of the literature analysis, it has been proved that coccoid forms of H. pylori are viable and their basic properties are similar to those of vegetative spiral forms (Andersen & Rasmussen, 2009). The lack of therapeutic success of our cases may be due to the fact that these bacteria, although viable, are as if in a dormant stage and therefore they cannot incorporate antibiotics, which consequently may prove ineffective. The microscopic examination of smeared biopsies is simple and is not time-consuming. In the presence of round organisms antibiotic treatment should be delayed or deferred in order to avoid therapeutic failure. This study finally suggests that to determine the presence of metronidazole-resistant strains, it is preferable to streak gastric biopsies onto solid media containing the chemotherapeutic at the threshold concentration rather than examine a few colonies from...
the primary plates. In this way, the entire biopsy bacterial population (300-5×10^5 bacteria) would be examined for its chemosusceptibility. The spontaneous mutation rate of such species for metronidazole resistance is very high (1×10^-5 organisms ca.), which means that metronidazole-resistant organisms are virtually always present in the stomach of almost all individuals. In addition, the technique employed can readily be applied to reveal resistances to the other antibiotics used in the treatment of *H. pylori* infection.

In conclusion, the factors that may modulate the outcome of *H. pylori* infection's eradicating treatment are numerous and the present study is far from complete. In addition to different therapeutic protocols, we did not consider the patients’ cytochrome P450 polymorphism, the possibility that resistance to metronidazole might be overcome with increased doses of drug, and that strains with different chemosusceptibility profiles can be simultaneously present in the same patient’s stomach (Grande et al., 2010). Our observations may help select appropriate treatment regimens. For example, PU and NUD patients should be considered independently in eradication trials and be managed differently in medical practice. The fact that infection by *H. pylori* strains with coccoid forms resulted in 100% eradication failure needs better analysis with a larger sample as this occurred in only 3.3% of the cases.

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**REFERENCES**


