Atrophic gastritis during acid-suppressive therapy with focus on ECL cell hyperplasia and intragastric bacteria

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Atrophic gastritis during acid-suppressive therapy

**with focus on ECL cell hyperplasia
and intragastric bacteria**

Silvia Sănduleanu, Maastricht 2001

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Atrophic gastritis during acid-suppressive therapy

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. dr. A.C. Nieuwenhuijzen Kruseman,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
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Imagination is sometimes more important than knowledge... knowledge is limited but imagination recycles the world. When I examine myself and my methods of thought, I come close to the conclusion that the gift of imagination has meant more to me than the talent for absorbing absolute knowledge.

Albert Einstein

Abbreviations

APAG	antral predominant atrophic gastritis
ABG	atrophic body gastritis
CPAG	corpus predominant atrophic gastritis
CgA	chromogranin A
ECL cell	enterochromaffin-like cell
H ₂ RA	histamine ₂ -receptor antagonist
PPI	proton pump inhibitor
<i>H.pylori</i>	<i>Helicobacter pylori</i>
non- <i>H.pylori</i>	non- <i>Helicobacter pylori</i>
IL	interleukin
GORD	gastro-oesophageal reflux disease

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Chapter 1

Introduction

Changes in gastric mucosa and luminal environment during acid-suppressive therapy: a review in depth

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Digestive and Liver Diseases (in press)

Introduction

The gastrointestinal effects of acid-suppressive therapy rank highly amongst the investigated issues in gastroenterology over the past two to three decades. Early in that period debates have focused on: (i) intragastric bacterial overgrowth and its possible consequences, such as enteric infections, alterations in nutrient absorption, and formation of potentially carcinogenic *N*-nitroso compounds (NOCs); (ii) hypergastrinaemia and the proliferation of gastric enterochromaffin-like (ECL) cells.

It is only within the last few years that substantial interest has been devoted to the relationship between gastric acid secretion and gastric infection with *Helicobacter pylori* (*H.pylori*): the *pH-Hp* interaction has inspired many studies, unraveling aspects of importance for both clinical practice and research. Herewith, we present an overview of the gastrointestinal effects of acid-suppressive therapy, with special attention to the impact of concurrent *H.pylori* infection on changes in gastric mucosa and luminal environment in this context.

Gastrin and enterochromaffin-like cell proliferation

The stomach comprises two regions with distinct anatomical and physiological conditions. The proximal two thirds of the stomach, known as the *body* and *fundic* region, contains acid-secreting parietal cells and histamine-releasing enterochromaffin-like (ECL) cells. The distal third of the stomach, known as the *antral* region, contains gastrin-producing G cells and somatostatin-producing D cells¹⁻³.

Intragastric acidity regulates the gastrin release from the antral G cells through a negative feedback mechanism: when the gastric juice pH falls due to high parietal cell secretion, the release of gastrin is inhibited, whilst when the gastric juice pH rises, hypergastrinaemia ensues to stimulate the parietal cell mass. Gastrin also exerts a powerful trophic effect on the ECL cells⁴⁻⁶. This explains why rats with marked hypergastrinaemia, as a consequence of profound acid suppression with proton pump inhibitors (PPIs) or with histamine₂-receptor antagonists (H₂RAs), may develop ECL cell hyperplasia and even ECL cell carcinoids⁷⁻¹¹. Similar changes in ECL cells have been documented in various other conditions associated with marked hypergastrinaemia, such as in rats subjected to partial fundectomy¹² or after exogenous infusion of gastrin¹³. On the opposite, the administration of the gastrin receptor antagonist PD 136450 prevented ECL cell hyperplasia¹⁴.

In humans, serum gastrin levels may increase up to four-fold in patients treated with omeprazole^{15,16}; this is accompanied by development of diffuse, linear, and/or micronodular ECL cell hyperplasia¹⁷⁻²¹. Furthermore, gastric ECL cell carcinoids can develop in severely hypergastrinaemic patients, in connection with disorders such as type A chronic atrophic gastritis or the Zollinger-Ellison syndrome associated to multiple endocrine neoplasia (MEN) type 1²²⁻²⁷. Taken together, these observations have raised concern that long-term profound acid suppression in humans may promote gastric endocrine tumour growth²⁸⁻³⁰.

Solcia *et al.*³¹ addressed this issue in a study of 448 patients with acid-related disease, who were treated with omeprazole for periods up to four years. They reported an increase in the prevalence of micronodular ECL cell hyperplasia from 2.5% at baseline to 10.4% at final biopsy; however, no ECL cell dysplasia or ECL cell neoplasia were observed. Lamberts *et al.*¹⁸ studied - both quantitatively and qualitatively - the ECL cell changes in 74 patients with acid-related diseases, treated with omeprazole 20-80 mg daily for up to five years. The ECL cell volume density increased from 0.36% of the total mucosa at baseline to 0.74% at 5 years; correspondingly, the percentage of patients with ECL cell hyperplasia increased from 36% at baseline to 67% at 5 years. Again, no ECL cell dysplasia or ECL cell neoplasia were observed.

Additional insights into the effects of acid-suppressive therapy on serum gastrin and gastric ECL cells have been gained after the discovery of *H.pylori*. In the presence of *H.pylori* infection, acid suppression results in a more marked gastrin elevation³²⁻³⁴ and a higher risk of developing ECL cell hyperplasia than in the non-infected subjects³⁵⁻³⁹. Eissele *et al.*³⁷ investigated 42 patients with reflux oesophagitis or peptic ulcer disease, who were treated with lansoprazole for periods up to five years. They found an increase in the prevalence of linear/micronodular ECL cell hyperplasia from 6.7% to 54.5% in *H.pylori*-positive subjects, compared to an increase from 0% to 7.7% in the *H.pylori*-negative subjects. The longest follow-up period has been reported in a study by Klinkenberg-Knol *et al.*³⁸ on 230 reflux oesophagitis patients, who were treated with omeprazole for a mean duration of 6.5 years (up to 11 years). The proportion of micronodular hyperplasia increased from 3% to 29% in the *H.pylori*-positive patients, compared to an increase from 3% to 11% in the *H.pylori*-negative group. Importantly, no premalignant or malignant ECL cell lesions were observed.

It can be concluded that available evidence now reassures the general safety of long-term (10 to 15 years) treatment with potent acid inhibitors in humans⁴⁰⁻⁴². However, the consequences of 'life-long' iatrogenic hypergastrinaemia on the ECL cells in humans are presently

not known, favouring caution in this respect^{43,44}. The recent report⁴⁵ of a patient who developed a gastric carcinoid tumour during long-term acid-suppressive therapy supports this attitude.

Non-*H.pylori* bacterial growth during acid-suppressive therapy

Most bacterial species do not survive at pH levels below 4; hence, the acidic gastric environment is considered the most important barrier against colonisation and infection by ingested bacteria⁴⁶⁻⁴⁹. It is well-established that low acid states resulting from pernicious anaemia⁵⁰⁻⁵², partial gastrectomy⁵³⁻⁵⁵, truncal vagotomy⁵⁶, or gastric cancer⁵⁷⁻⁶⁰, all predispose to colonisation of the stomach by aerobic and - to a minor degree - by anaerobic, gram-positive and gram-negative bacteria. Pooled data from studies in individuals with natural or artificial hypochlorhydria have documented a logarithmic relationship between intragastric pH and median bacterial counts⁶¹. Additionally, these studies have brought into focus potential consequences of the intragastric bacterial overgrowth, such as: (i) increased susceptibility for enteric infections; (ii) alterations in nutrient absorption; and (iii) formation of potentially carcinogenic *N*-nitroso compounds.

Enteric infections

Intragastric hypochlorhydria increases the risk of enteric infections with bacterial agents (e.g. *V.cholerae*, species of *Shigella* and *Salmonella* or *Clostridium difficile*), or with parasitic agents (e.g. giardiasis, strongyloidiasis, amoebiasis)⁶²⁻⁶⁴. In a large population-based study, subjects taking acid-suppressive medication had a three-fold higher risk of developing bacterial diarrhoea than those taking antihypertensive or antiasthmatic medication. This effect was related to the degree of gastric acid inhibition (RR, omeprazole: 5.0; ranitidine: 3.7; cimetidine: 1.8)⁶⁵. In a case-control study, treatment with acid inhibitors in the previous month was associated with an increased risk for *Salmonella*-infections (RR, 4.6), and *Campylobacter*-infections (RR, 11.7); such increased susceptibility was confined to the elderly subjects^{66,67}.

Altered nutrient absorption

Much scientific effort has also been directed to the interrelation between bacterial overgrowth and alterations in the metabolisms of nutrients, minerals, and vitamins during natural and artificial hypochlorhydria and achlorhydria⁶⁸⁻⁷².

In a series of seventeen healthy, elderly subjects with confirmed bacterial overgrowth - either due to atrophic gastritis or to omeprazole treatment (40 mg daily, 10 days), the 72-h faecal fat levels - the index for fat malabsorption - were within the normal range⁷³.

Short-term treatment with omeprazole was also without significant effects on the absorption of dietary calcium, phosphorus, magnesium, or zinc in a study involving healthy volunteers⁷⁴. Serum iron and ferritin concentrations have been monitored in 34 patients with peptic ulcer disease receiving omeprazole therapy up to four years; no significant decrease in their concentrations was found⁷⁵.

However, current data suggest that acid suppression may impair the absorption of protein-bound vitamin B₁₂ (cobalamin)⁷⁶⁻⁸⁰. In a study of 34 patients receiving long-term omeprazole therapy, serum vitamin B₁₂ levels remained stable and within the normal range during the first three-year of follow-up; interestingly, a longer duration of acid-inhibitory treatment resulted in a slight but significant downward trend in serum vitamin B₁₂ levels, although this was not confirmed by other investigators^{75,77,81}.

N-nitrosamine formation

An additional issue regarding the intragastric bacterial growth was related to the ability of some species to convert ingested nitrates to nitrites and *N*-nitrosamines⁸²⁻⁸⁴. In fact, formation of NOC may occur spontaneously in the acidic stomach (chemical nitrosation), or at neutral pH (bacterial nitrosation)^{85,86}. The NOCs are mutagenic and carcinogenic in experimental animal models and have been suggested to play a role in the gastric carcinogenesis in humans⁸⁷⁻⁹⁰. Hence, these observations have prompted investigations to determine whether the intragastric bacterial growth during antisecretory therapy enhances the production of NOCs, and may thereby increase the risk of gastric cancer. Studies have been conducted in healthy volunteers and in patients with different acid-related conditions, thusfar, with conflicting results.

Stockbrügger *et al.*⁹¹ investigated 23 patients with peptic ulcer disease, who received a six-week course of cimetidine, 1 g daily. They reported a significant increase in intragastric aerobic and anaerobic bacteria and subsequently elevated gastric juice *N*-nitrosamines concentrations. Similar conclusions were drawn by Sharma *et al.*⁹² in a study of 10 healthy volunteers, who received a two-week course of omeprazole 30 mg daily. On the contrary, Verdú *et al.*⁹³, in a study of 14 healthy volunteers receiving a two-week course of omeprazole 20 mg daily, reported increased bacterial concentrations (compared to a placebo-group), but no differences in nitrates, nitrites, or *N*-nitrosamines concentrations. Also, Thorens *et al.*⁹⁴ investigated 47 patients with peptic ulcer disease, who received a four-week course of either omeprazole 20 mg daily, or cimetidine 800 mg daily. Increased intragastric bacterial counts were found, but no changes in the concentrations of nitrates, nitrites, and *N*-nitrosamines.

Several explanations could account for the dichotomy of results: First, it is known that the volatility of many NOCs makes it difficult to accurately quantify them; it can be reasoned that methodological aspects, such as differences in sample collection, storage, and analysis, could bring about different results. A second explanation could reside in differences in the degree of acid suppression achieved with the various regimens, as well as in the proportion of nitrate-reducing bacteria. Thirdly, recent data suggest that concurrent *H.pylori* infection may play a role in the intragastric *N*-nitrosation during acid suppression. As such, in *H.pylori*-negative subjects, short-term administration of either H₂RAs or PPIs consistently increased the intragastric bacterial counts, but did not increase the gastric juice nitrates, nitrites, and *N*-nitroso compounds^{95,96}. On the opposite, in a study by Mowat *et al.*, *H.pylori*-positive subjects treated with a PPI had greater elevation of intragastric pH and a 100-fold greater degree of colonisation by non-*H.pylori* bacteria than the *H.pylori*-negative subjects. Additionally, the intragastric *N*-nitrosamine formation was more marked in the *H.pylori*-positive treated subjects compared to the *H.pylori*-negatives^{97,98}. At the first glance, the latter findings appear paradoxical, as *H.pylori* is known to have low nitrosation capacity⁹⁹. It should be remembered, however, that chronic inflammation increases the production of nitric oxide (NO), which in turn can lead to nitrosation of amines¹⁰⁰⁻¹⁰². Hence, it is tempting to hypothesize that the enhanced *N*-nitrosamine formation in *H.pylori*-infected subjects treated with acid suppression may result from nitrite production by the *H.pylori*-activated inflammatory cells, combined with nitrite production by the other (non-*H.pylori*) bacterial species.

***Helicobacter pylori* gastritis and gastric acid secretion**

Helicobacter pylori infection elicits chronic-active inflammation of the gastric mucosa in nearly all colonised subjects¹⁰³⁻¹⁰⁶. Despite a vigorous immune response, the bacterium is not cleared spontaneously, and infection persists lifelong. The clinical outcome of the *Helicobacter*-associated disease is driven by an interplay between bacterial pathogenicity, host response, and environmental factors^{107,108}.

McColl *et al.* have described a 'two-way interaction' between *H.pylori* gastritis and gastric acid secretion^{109,110}. This is well-reflected during administration of acid-suppressive medication. It is known that *H.pylori*-positive subjects treated with acid suppression have higher intragastric pH than the *H.pylori*-negatives^{32-34,111-114}. There are several reasons for this phenomenon: *H.pylori* infection causes body gastritis, which impairs parietal cell secretion¹¹⁴⁻¹¹⁶. Additionally, body gastritis is associated with loss of M₃ muscarinic receptors, further altering the gastric acid secretory function¹¹⁷. Also, *H.pylori* generates ammonia, with subsequent buffering of the intragastric acid¹¹⁸. Infection with *H.pylori*

may stimulate the production of interleukin (IL)-1 β and nitric oxide, which are recognised inhibitors of the gastric acid secretion¹¹⁹⁻¹²¹. *In vitro*, *H.pylori* releases acid-inhibitory substances, albeit their impact on the *in vivo* acid secretion remains unclear¹²²⁻¹²⁴.

On the other hand, gastric acid secretion modulates the ecology and density of gastric colonisation by *H.pylori*, influencing the topographic subtype, extent, and severity of gastritis¹²⁵⁻¹²⁸. In *hypersecretors*, *H.pylori* colonisation and the subsequent chronic inflammation predominate in the antrum; these subjects are at risk of developing duodenal ulcer disease. In contrast, the *hyposecretors* are characterised by a body predominant pattern of gastritis, which results in impaired parietal cell function; the progression of inflammation causes destruction of the oxyntic glands, further compromising the gastric acid output, and eventually leading to chronic hypochlorhydria, and an increased risk of non-cardia gastric cancer.

Classification of chronic gastritis

Several classifications have been used to describe chronic gastritis¹²⁹⁻¹³⁸. In 1973, Strickland & Mackay have proposed a topographic classification into three categories: the A-type (corpus alone), B-type (antrum alone), and AB-type (both antrum and corpus)¹²⁹. The classification by Correa¹³⁰ has suggested the division in diffuse antral-predominant gastritis and multifocal atrophic gastritis, which was later subdivided into non-ulcer pangastritis and progressive intestinalised metaplasia¹³⁸. As a strength, these classifications correlated the topography of chronic gastritis to the distinct functional consequences (e.g. the aforementioned effects on gastric acid secretion), and hence, to the expected clinical outcome. As a pitfall, they did not score atrophy and inflammation as distinct parameters, nor did they grade the severity of these items.

In the era of *H.pylori*, tighter guidelines for the diagnosis of chronic gastritis have been defined. The Sydney classification for the histological assessment of chronic gastritis - up-dated in 1994 - is presently used as the standard in clinical practice and research¹³⁴⁻¹³⁷. This classification is also subject of ongoing debates, mainly related to dissent on definition of gastric gland atrophy and inter-observer variations in the assessment of histopathological features of chronic gastritis^{139,140}. At the recent *Atrophy 2000 Meeting*, the definition of atrophy was clarified as 'absence of appropriate glands' (e.g. mucus glands in the gastric antrum and oxyntic glands in the gastric body)¹⁴¹. The following major aspects have been emphasized: (i) gastric glands must be unequivocally 'absent', as opposed to 'displaced' and 'invisible only' because of concomitant infiltration of the lamina propria with inflammatory cells; in this latter situation, the term 'indefinite for atrophy'

can be used as a temporary category, until the follow-up allows final judgement; (ii) any replacement of the normal glands by 'fibrosis and/or metaplastic epithelium' is equivalent to atrophy. Finally, it was agreed that the accurate diagnosis of gastric gland atrophy requires examination of multiple specimens, taken from both the antrum and the body mucosa. For research purposes, a single pathologist should review the histological material, in a blinded fashion, in order to minimize inter-observer bias.

***H.pylori* gastritis and acid-suppressive therapy**

H.pylori gastritis usually predominates in the antrum and decreases gradually over the antral-body transitional zone¹⁴². This pattern of colonisation is due to the optimal pH that enables bacterial survival and growth. *H.pylori* is a neutrophil organism, with a recognised acid tolerance; this is attributable to the activity of *urease* enzyme, which is driven by a complex gene cluster¹⁴³⁻¹⁴⁷. *In vitro*, in the presence of urea, the optimal growth of *H.pylori* occurs at a pH level between 4.5 and 6^{143,144}. Under physiological conditions, *H.pylori* colonisation predominates in the antrum. Treatment with acid-suppressive medication elevates the antral pH, impairing the local growth conditions for *H.pylori*. As a consequence, a bacterial 'shift' occurs from the antrum to the body mucosa, with inversion in the gastritis pattern (from antral predominant gastritis to body predominant gastritis), and subsequent progression to body gland atrophy^{35,148-151}. This observation originates, in fact, from older studies in patients with low-acid states, like gastric ulcer disease or following surgical vagotomy; in such cases an accelerated progression to atrophic gastritis - at an annual rate of 4-8% - has been observed¹⁵²⁻¹⁵⁴. This was in contrast to the 1-3% annual rates of progression to atrophic gastritis reported in general populations¹⁵⁵⁻¹⁵⁸. A similar development was then noticed in patients treated with different types of antisecretory drugs, including H₂RAs and antacids^{159,160}; however, this phenomenon has been thoroughly studied only after the introduction of profound inhibitors of gastric acid secretion, namely the PPIs^{10,19,20,31,35-39,161-168}. Evidence now suggests that this phenomenon occurs within a few weeks to months after the start of treatment¹⁶⁹, and is reversible after the recovery of acid secretion^{149,170}. As atrophic body gastritis is a recognised precursor lesion for the non-cardia gastric cancer, concern and speculation have emerged regarding the potential risk for development of gastric cancer in this setting^{171,172}.

Table 1.1 presents a chronological chart of the clinical studies investigating the gastric mucosal histology during treatment with different antisecretory drugs. Solcia *et al.*³¹ observed the development of atrophic gastritis in 12% out of 202 patients with reflux disease, who were treated with omeprazole for a mean period of 1.5 years.

Table 1.1. Clinical studies investigating chronic gastritis during acid-suppressive therapy.

Authors & Journal	Study design	Study population	Dose & (mean) duration of therapy	Results & comments*
Solcia et al, <i>Digestion</i> 1992 ²¹	Open, unrandomised, compassionate-use	202 GORD patients	OME 20-40 mg daily, 1.5 years	Annual increase in body atrophy: 8% *Patients not stratified for Hp status
Lamberts et al, <i>Gastroenterology</i> 1993 ¹⁹	Open, unrandomised, compassionate-use	74 GORD or PUD patients	OME 20-40 mg daily, Up to 5 years	Annual increase in body atrophy: 6% *Patients not stratified for Hp status
Klinkenberg-Krol et al, <i>Ann Intern Med</i> 1994 ²⁸	Open, unrandomised, compassionate-use	91 GORD patients	OME 20-40 mg daily 48(36-64) months	Annual increase in body atrophy: 6.25% *Patients not stratified for Hp status
Kuipers et al, <i>NE/IM</i> 1996 ⁶⁸	Open, unrandomised, compassionate-use	177 GORD patients treated with ARS (n=72) or OME (n=105)	OME 20-40 mg daily, 5 (3-8) years	Annual increase in body atrophy: Hp(+) ARS group: 0% (97.5%CI: 0-2.2) Hp(+) OME group: 6.1% (95% CI: 3.8-8.8) Hp(-) OME group: 0.8% (95%CI: 0.2-3) Annual increase in body atrophy: Hp(-) group: 1-3% per yr
Eiselle et al, <i>Gastroenterology</i> 1997 ³⁷	Open, unrandomised, compassionate-use	38 patients with GORD or PUD	LANSO 15-30 mg daily 5 years	Hp(+) group: 9.9% per yr for the first 2 yrs, then 2.4% per yr for the next 3 yrs Increase in the degree of active and chronic body gastritis in Hp(+) patients; no change in atrophy
Berstad et al, <i>Gut</i> 1997 ⁶⁹	Prospective, randomised	90 GORD patients	LANSO 15-30 mg daily 64 (15-74) weeks	Increase in the degree of active body gastritis with both regimens;
Meining et al, <i>Aliment Pharm & Ther</i> 1997 ⁶⁰	Double-blind, randomised, comparative study	PUD patients treated with antacids (n=67) or RANI (n=71)	Antacids: AlMg(OH) ₂ ; 280mval daily or RANI 300 mg daily, 4 weeks	Increase in the degree of active body gastritis with both regimens;
Stolte et al, <i>Aliment Pharm & Ther</i> 1998 ⁶²	Prospective, randomised, comparative study	111 Hp(+) GORD patients	LANSO 15 mg daily (n=33), or 30 mg daily (n=35), or OME 20 mg daily (n=43) 12 months	Increase in the degree of chronic body gastritis in Hp(+) patients, in both LANSO group and OME group; no change in atrophy
Diebold et al, <i>Dig Dis Sci</i> 1998 ⁶⁷	Cross-sectional, case-control	319 GORD patients, treated with OME (n=201) or RANI (n=118), and non-treated controls (n=215)	OME 20-40 mg daily, 23±14 months RANI 150-300 mg daily, 35.7±3.7 months	Prevalence of body atrophy in Hp(+) patients: OME group: 82% RANI group: 93% controls: 88%
Lundell et al, <i>Gastroenterology</i> 1999 ⁶⁶	Prospective, randomised	299 GORD patients treated either with ARS (n=144) or OME (n=155)	OME 20-40 mg daily 3 years	Annual increase in body atrophy: Hp(+) ARS group: 3.8% Hp(+) OME group: 8.6%
Moayyedi et al, <i>Helicobacter</i> 2000 ⁶³	Double-blind, prospective, randomised	Hp(+) GORD patients treated with OME alone (n=20) or OME and eradication (n=21); and Hp(-) GORD patients treated with OME (n=20)	OME 20-40 mg daily, 1 year	Body atrophy developed in 46% of the Hp(+) OME alone group 0% of the Hp(+) OME and eradication group 0% of the Hp(-) OME group

Ranitidine (RANI), Omeprazole (OME); Lansoprazole (LANSO)

These changes were paralleled by an increase in the prevalence of micronodular ECL cell hyperplasia. In a study by Lamberts *et al.*¹⁹, 19% out of 74 patients with either reflux diseases or peptic ulcer disease, treated with omeprazole for periods up to five years developed atrophy of the body mucosa.

In this context, the scientific interest shifted from the less harmful gastric ECL cell lesions towards the yet incompletely characterised behaviour of chronic gastritis during treatment with acid-suppressive medication. It became clear that further studies on this topic had to stratify for the *H.pylori* status of the subjects, and to use proper control populations as reference. In this sense, the study by Kuipers *et al.*³⁶ offered major insights into this field. The authors have followed-up two GORD cohorts: one (n=105 patients) was treated with omeprazole, and the other (n=72 patients) was treated with fundoplication. In *H.pylori*-negative patients, the annual rate of progression to atrophic gastritis was less than 2% in both cohorts. The logical interpretation was that the development of gland atrophy *per se* cannot be attributed to the long-term treatment with PPIs alone. Similarly, in *H.pylori*-positive patients treated with fundoplication, nearly no atrophy developed during the follow-up. In contrast, in *H.pylori*-positive patients treated with omeprazole the body gastritis exacerbated gradually, and after five years of treatment one-third of these patients ended-up with body mucosal atrophy. The annual incidence of atrophic gastritis was 6.1% in this latter subgroup, significantly higher than in the *H.pylori*-positive subjects treated with fundoplication. It should be mentioned, however, that intestinal metaplasia developed in a minority of these cases only, and that it was almost exclusively of type I. Three other cohort studies in patients treated with lansoprazole confirmed the aggravation of body gastritis during acid inhibition^{37,161,162}. Among them, the study by Berstad *et al.*¹⁶¹ reported increased epithelial cell proliferation in the body mucosa of *H.pylori*-positive patients treated with lansoprazole, paralleled by an increasing degree of chronic-active inflammation. However, some other studies did not support this hypothesis¹⁶⁶⁻¹⁶⁸. The most important evidence in this sense comes from a study by Lundell *et al.*¹⁶⁶, in which body mucosal atrophy developed in 17.9% out of 39 *H.pylori*-positive GORD patients treated with omeprazole, compared to 4.5% out of 44 *H.pylori*-positive GORD patients treated with surgical fundoplication. As this difference did not achieve statistical significance, they claimed a 'lack of progression to atrophic gastritis in *H.pylori*-positive patients on omeprazole'. This conclusion triggered heated debates in the medical press, and criticism pointed towards a possible type 2 error of Lundell's study¹⁷³⁻¹⁷⁷. Additionally, the pre-treatment and/or post-treatment with omeprazole or vagotomy of patients in the

antireflux surgery arm could have biased the results and the conclusion of this study.

It can be concluded that the aggravation of body gastritis in *H.pylori*-positive patients treated with acid suppression is a consistent observation in most of the available studies. This favours the present concept that '*H.pylori* test-and treat strategy' is advisable prior to the start of long-term acid-suppressive therapy in order to prevent the accelerated development of atrophic gastritis in this setting^{163,178}.

Atrophic gastritis during acid-suppressive therapy: pathophysiological aspects

Since the recognition of gastric atrophy in 1870 - in postmortem samples from a patient with pernicious anaemia¹⁷⁹ - much attention and research interest have been devoted to decipher its underlying pathophysiology. There is presently no doubt that infection with *H.pylori* is a major cause of chronic atrophic gastritis. The more virulent *cagA*-positive *H.pylori* bacterial strains induce a more severe gastritis and a more rapid progression to glandular loss than the *cagA*-negative strains¹⁸⁰⁻¹⁸³. Importantly, recent evidence highlights the critical role of host genetic factors, such as abnormal variants of IL-1 β gene, in the development of atrophic gastritis and gastric cancer in *H.pylori*-positive individuals¹⁸⁴. The contribution of the environmental factors (e.g. salt, micronutrients and vitamins intake, alcohol consumption, and smoking) to this development, although long-recognised, remains heretofore ill-defined^{130,185-187}. Among these environmental factors, treatment with acid-suppressive medication is known to induce complex changes in the intragastric environment, which encompass both the *luminal* and the *mucosal* compartment. Their characterisation is of great importance, as it may provide the rationale for the observed rapid development of atrophic gastritis in this context.

It has been shown that *H.pylori* colonisation density of the body mucosa does not change significantly during acid suppression^{35,163}. This suggests that the aggravation of chronic body gastritis may depend on *H.pylori* infection as well as on additional, yet unknown factors.

Which effects has gastric acid suppression on the intragastric milieu in the presence of *H.pylori* infection?

- Decreased gastric acid secretion favours a deeper location of *H.pylori* in the mucosal foveolae; it also hampers the dilution and washout of the cytotoxic and pro-inflammatory agents induced by *H.pylori* infection, which in turn may cause mucosal damage^{37,188-192}. Under normal acidic conditions, the ammonia produced by *H.pylori* is ionized, being unable to penetrate through the epithelial cells, whilst at high intragastric pH, ammonia remains unionized and may exert toxic effects. Additionally, chemical alterations of bacterial toxins in a less acidic environment could aggravate mucosal damage^{163,189}.
- *H.pylori*-positive subjects treated with acid suppression have greater increases of intragastric pH than the *H.pylori*-negative subjects. This chronic gastric hypochlorhydria predisposes to colonisation by non-*H.pylori* bacterial species, and subsequently increased nitrite concentrations, and decreased vitamin C in the gastric juice may occur⁹⁸. Additionally, intragastric bacteria may produce alcoholdehydrogenase, which in turn promotes the synthesis of acetaldehyde from alcohol^{193,194}. All these biochemical alterations are deemed to have mutagenic and carcinogenic effects.
- Antigenic mimicry has been described between *H.pylori* and gastric mucosa, and such autoimmune phenomena may result in development of body atrophic gastritis¹⁹⁵⁻¹⁹⁸. By analogy with pernicious anaemia, it is tempting to speculate that chronic hypochlorhydria may enhance and perpetuate these changes, through yet unknown mechanisms.
- *H.pylori*-positive subjects treated with acid suppression also have elevated gastrin levels, which are 30-50% greater than in *H.pylori*-negative subjects. The magnitude of the hypergastrinaemic response is also driven by host factors, such as CYP2C19 polymorphism¹⁹⁹. Recent experimental data suggested that hypergastrinaemia, in itself, may contribute to the development of gastric gland atrophy²⁰⁰. Transgenic mice with moderate hypergastrinaemia showed progression to atrophic gastritis and gastric cancer, probably through activation of several growth factors. Furthermore, the concurrent infection with *H.pylori* accelerated this process.

Gastric mucosa and luminal environment during acid-suppressive therapy:

What do we know on this topic?

- *During long-term acid suppression, gastric fundic ECL cell hyperplasia may develop, especially in the presence of H.pylori infection. So far, in humans, no neoplastic changes directly attributable to pharmacological acid inhibition have been demonstrated.*
 - *Acid-suppressive therapy increases the risk of enteric infections.*
 - *Acid-suppressive therapy does not alter fat and mineral bioavailability, but it may decrease the absorption of protein-bound vitamin B₁₂.*
 - *Acid suppression invariably results in overgrowth of non-H.pylori bacteria; the concurrent infection with H.pylori may facilitate intragastric formation of N-nitrosamines.*
 - *The clinical outcome of H.pylori gastritis is strongly influenced by the individual gastric acid secretory status: in hypersecretors, gastritis predominates in the antrum, and may lead to duodenal ulcer disease; in hyposecretors, gastritis predominates in the body mucosa, and may lead to gland atrophy, chronic hypochlorhydria, and risk of gastric cancer.*
 - *Acid-suppressive therapy alters the H.pylori gastritis, transforming the antral-predominant pattern into a body-predominant pattern. This phenomenon may occur during treatment with any type of antisecretory drug.*
 - *The aggravation of chronic body gastritis during acid suppression is confined to the H.pylori-positive subjects. So far, the underlying pathophysiology remains unclear.*
 - *Eradication of H.pylori prior to the start of acid-suppressive therapy may prevent the aggravation of chronic body gastritis in this setting.*
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Chapter **2**

Aims and outline of the thesis

Aims and outline of the thesis

Currently, there is a large body of information regarding the gastrointestinal effects of acid-suppressive therapy. Most of these studies examined hypergastrinaemia and ECL cell proliferation; intragastric bacterial overgrowth and its consequences; and development of atrophic gastritis in the presence of *H.pylori* infection as distinct issues. Hitherto, the interrelation between the aforementioned phenomena has received little attention.

The research described in this thesis focuses on the development of chronic atrophic gastritis and proliferation of the ECL cells during treatment with acid-suppressive medication. Particular attention was paid to the role of intragastric *H.pylori* and non-*H.pylori* bacteria in this development. The clinical utility of serological markers for these conditions was also investigated.

We addressed the following issues:

- Does medium- and long-term treatment with acid-suppressive medication result in stimulation of the gastric ECL cell population? If so, is serum chromogranin A (CgA) a useful marker to monitor these changes?
- Is there a relationship between gastric luminal and mucosal overgrowth of non-*H.pylori* bacteria during acid-suppressive therapy, and which factors influence their occurrence?
- Is the gastric double infection with *H.pylori* and non-*H.pylori* bacteria during acid-suppressive therapy associated with an increased risk for development of atrophic gastritis? If this is the case, could such development be due to an enhanced pro-inflammatory cytokine response?
- Is serum pro-inflammatory cytokine profile of additional value to serum gastrin, and serum pepsinogens measurement in screening for atrophic body gastritis?

To answer these questions, we have investigated a series of 230 consecutive dyspeptic patients referred for upper gastrointestinal endoscopy to the open-access unit of the University Hospital Maastricht, between January 1997 and June 1998. They were patients with gastro-oesophageal reflux disease, receiving acid-suppressive medication, and non-treated dyspeptic controls, with normal endoscopic findings.

Chapter **3**

Serum gastrin and chromogranin A during medium- and long-term acid suppressive therapy: a case-control study

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Abstract

Background & Aim

Serum chromogranin A (CgA) is regarded as a reliable marker of neuroendocrine proliferation. We previously described increased serum CgA levels during short-term profound gastric acid inhibition. In the present study we investigated serum gastrin and CgA levels in dyspeptic patients during continuous medium (6 weeks to 1 year)- or long-term (1 year to 8 years) gastric acid suppressive therapy.

Patients & Methods

114 consecutive dyspeptic patients referred for upper GI-endoscopy were enrolled in a cross-sectional, case-control study [62 patients on continuous antisecretory therapy, either with proton pump inhibitors (PPIs) (n=47) or H₂-receptor antagonists (H₂RAs) (n=15) for gastro-oesophageal reflux disease with or without Barrett's oesophagus or functional dyspepsia, and 52 age- and sex-matched patients without medical acid inhibition and with normal endoscopic findings (control group)]. Doses of omeprazole ranged from 20 mg to 80 mg daily and ranitidine from 150 mg to 450 mg daily. Fasting serum CgA and serum gastrin levels were measured by radioimmunoassay (reference values: serum CgA <4.0 nmol/l; serum gastrin <85 ng/l). *H.pylori* serology was assessed by Western blotting.

Results

Fasting serum CgA levels positively correlated with serum gastrin in the entire study population ($r=0.55$, $P=0.0001$). Median serum CgA values were higher in patients treated with PPIs vs H₂RAs [2.8 (2-5.9) nmol/l vs 2 (1.9-2.3) nmol/l, $P<0.002$] and controls [2.8 (2-5.9) nmol/l vs 1.8 (1.5-2.2) nmol/l, $P<0.0001$] and did not differ between patients treated with H₂RAs and controls. Serum gastrin and CgA levels in patients on PPI therapy positively correlated with the degree and duration of acid inhibition. Patients on long-term PPI therapy had significantly higher fasting serum gastrin and CgA than those on medium-term PPI therapy [127 (73-217) ng/l vs 49 (29-78) ng/l, $P<0.0001$ and 4.8 (2.8-8) ng/l vs 2.1 (1.9-2.6) ng/l, $P<0.001$]. No such relation was found in patients on medium- vs long-term H₂RAs. Overall, patients with positive *H.pylori* serology had higher serum gastrin and CgA levels than those with negative *H.pylori* serology [51 (27-119) ng/l vs 27 (14-79) ng/l, $P=0.01$; 2.4 (1.9-3.4) nmol/l vs 2.0 (1.7-2.5) nmol/l, $P=0.05$].

Conclusions

During long-term continuous PPI treatment, serum gastrin and CgA levels are significantly elevated compared to H₂RA treatment and non-treated dyspeptic controls. *H.pylori* infection seems to affect gastric ECL cell secretory function. Increased serum CgA values during long-term profound gastric acid inhibition could reflect either gastric enterochromaffin-like cell hyperfunction or proliferative changes.

Introduction

It is widely accepted that profound acid inhibition leads to antral gastrin-producing (G) cell hyperplasia and hypergastrinaemia^{1,2}. Gastrin has a specific effect on the enterochromaffin-like (ECL) cell, regulating its function and promoting its growth³⁻⁵. In rats, gastric carcinoids may occur after long-term acid suppression⁶⁻¹⁰. However, in humans, the development of gastric ECL cell carcinoids in response to chronic hypergastrinaemia has been restricted to patients with the Zollinger-Ellison syndrome (ZES) as a part of multiple endocrine neoplasia (MEN) - type I¹¹⁻¹³ or with chronic atrophic gastritis type A (A-CAG)¹⁴⁻¹⁷, pointing towards the involvement of additional, and yet unidentified factors. The genomic events associated with MEN-I-ZES, e.g. the loss of a suppressor oncogene in 11q13 or the intragastric environmental changes due to achlorhydria in type A CAG, might explain gastric endocrine cell proliferation in these particular situations¹⁸⁻²⁰.

Well-documented prospective studies, focused on the safety of long-term profound medical acid inhibition, do not substantiate the risk of carcinoid tumour formation²¹⁻²⁴. Recently, the combined effects of profound gastric acid inhibition and *Helicobacter pylori* (*H.pylori*) infection on serum gastrin levels have elicited research interest²⁵⁻²⁸. In a follow-up study designed to assess serum gastrin levels and gastric endocrine and non-endocrine mucosal changes during five-year treatment with lansoprazole, *H.pylori* infection was regarded as a risk factor for the progression of fundic gastritis and the development of ECL cell hyperplasia²⁹.

Taking into consideration the slow rate of progression from hyperplasia to dysplasia and to carcinoid tumour formation, it seems premature to conclude that ECL cell proliferation due to pharmacologically induced hypergastrinaemia is an innocuous phenomenon and that clinical surveillance is unnecessary. Moderate gastrin elevations exert constant trophic effect on the ECL cells and the clinical consequences of this over many years warrant further consideration³⁰⁻³².

The identification of a noninvasive, accurate biochemical marker of the ECL cell function and growth is therefore an important issue for clinical practice and research^{30,33}. Recent studies have described serum chromogranin A (CgA) as a reliable marker of neuroendocrine cell proliferation³⁴⁻³⁸. Increased serum CgA concentrations have been reported in patients with gastrinoma³⁹⁻⁴⁰, as well as in patients with type A autoimmune gastritis⁴¹. Waldum *et al.* described elevated serum CgA levels after short-term treatment with omeprazole, suggesting its

possible use as a test to evaluate ECL cell hyperplasia in patients with hypergastrinaemia secondary to acid inhibition⁴². Our group has also shown that after short-term medical acid inhibition with three different drug regimens (ranitidine 300 mg b.d., omeprazole 20 mg o.m., omeprazole 40 mg o.m.) in healthy volunteers, serum gastrin and chromogranin A increase in relation to the degree of acid inhibition and that they are strongly correlated⁴³.

The present study was performed to investigate the relationship between serum gastrin and CgA levels during medium- and long-term profound acid inhibition. The influence of *Helicobacter pylori* infection on serum gastrin and chromogranin A levels, in these circumstances, was evaluated as well.

Subjects and methods

Subjects

One hundred and fourteen consecutive dyspeptic patients (F=52, M=62) referred for open-access upper gastrointestinal endoscopy between January and August 1997 were enrolled in a cross-sectional, case-control study. All subjects had given informed consent prior to inclusion. The mean age was 50 years, with a range of 19-75 years. The study group consisted of 62 patients on continuous medium- (6 weeks to 1 year) or long-term (1 year to 8 years) acid suppressive therapy for gastro-oesophageal reflux disease (GORD) with or without Barrett's oesophagus, or for functional acid dyspepsia. The control group consisted of 52 dyspeptic patients with similar distribution of age and gender, who did not receive acid suppressive medication or antacids before referral and who had normal endoscopic findings.

Patients with active or previous peptic ulcer disease, those who had received *H.pylori* eradication therapy or had undergone previous gastric surgery / vagotomy were not included in the study population. Patients with other gastrointestinal or pancreatic diseases, with known malignancies, endocrine or autoimmune diseases, liver or kidney disorders were not eligible.

The study was approved by the medical ethical committee of the University Hospital Maastricht.

Study design

After informed consent, an extensive medical history was obtained in all patients and the clinical symptoms, type and daily dose of medication, duration of continuous acid inhibition in the recent years, regularity of

drug use and eventual comorbidity / comedication or family history of gastric cancer were rigorously recorded.

Endoscopy was performed after an overnight fast with standard gastroscopes (Pentax EG-2901). At the beginning of the endoscopy, a 5-10 ml sample of gastric juice was obtained through the suction channel of the endoscope into a trap placed in the suction line. Fasting gastric juice pH was immediately measured, using pH-paper strips with grading steps of 0.5, from pH 0 to pH 14 (Schleicher & Schüll GmbH; Dassel, Germany)⁴⁴. A 10 ml venous blood sample was collected from all subjects for the assessment of fasting serum gastrin, serum chromogranin A and *H.pylori* serology.

Determination of serum gastrin levels

Fasting blood samples were immediately centrifuged and the sera were stored at -80°C until further analysis. The serum gastrin concentration was measured by a radioimmunoassay method using an antibody raised in a rabbit against synthetic unsulphated human gastrin 2-17 covalently coupled to bovine serum albumin as previously described⁴⁵. The antibody binds to all known circulating gastrin fragments. The upper limit of the normal range for fasting state was 85 ng/l.

Determination of serum chromogranin A levels

A sensitive and specific radioimmunoassay method was used for the determination of chromogranin A levels in the serum samples^{35,36}. Polyclonal antibodies raised in rabbits against a fragment of chromogranin A (CgA 116-439) purified from the urine of a patient with carcinoid tumour were used in the assay. Based on a group of healthy individuals, the upper limit of the normal range for fasting serum chromogranin A levels was 4 nmol/l.

Helicobacter pylori serology

A commercial Western blotting for IgG (Helicoblot 2.0, Imphos BV, Zambon Group, Amersfoort, The Netherlands) was used for serological assessment of *H.pylori*. Bands were present for 19.5, 26.5, 30, 35, 89 (vacA), and 116 kD (cagA) proteins. The patients were considered to be positive if two bands of the 19.5, 26.5, or 30 kD protein or any of the bands among the 35, 89, or 116 kD proteins were positive. *H.pylori* positive and *H.pylori* negative control sera were included at each determination.

Statistical evaluation

Statistical analysis was performed using the SPSS software package. As the data were not normally distributed, values were expressed as medians, with 25-75% quartile ranges and group comparisons were performed using non-parametric tests. The Kruskal-Wallis test was used for multiple comparisons and the Mann-Whitney U test for two group comparisons of continuous variables. Differences in dichotomous variables were evaluated using the χ^2 -test or the Fisher's exact test, depending on the number of patients. Correlations were performed with the Spearman rank test. In addition, linear regression analysis was used to assess the relationship between serum gastrin, CgA, and the duration of PPI therapy. Two-sided P-values <0.05 were assumed to indicate statistical significance.

Results

Study population

Table 3.1 illustrates the clinical features of the study population, subdivided according to acid inhibition regimen. There was a similar distribution of age and gender among the three subgroups of patients. Omeprazole doses ranged from 20 mg to 80 mg daily and of ranitidine from 150 mg to 450 mg daily. The median length of acid inhibition therapy in the group receiving H₂RAs (n=15) was 2.4 years (range 6 weeks-10 years) and in patients on PPIs (n=47) was 1.8 years (range 6 weeks-8 years). In patients on long-term PPIs (n=26) the median duration on therapy was 2.8 years (1-8 years). Eight of these 26 patients (30%) were continuously treated for 1 to 2 years, 7 (27%) for 2 to 3 years, 5 (20%) for 3 to 4 years, and 6 (23%) for more than 4 years. In patients on long-term H₂RAs (n=8) the median duration on therapy was 3.2 years (1-10 years). Two (25%) of them received continuous therapy with H₂RAs for 1 to 2 years, 2 (25%) for 2 to 3 years, 3 (38%) for 3 to 4 years, and 1 (12%) for about 10 years.

Table 3.1. Clinical characteristics of the study population subdivided according to the regimen of acid inhibition.

Characteristics	Control group (n=52)	H ₂ RA group (n=15)	PPI group (n=47)
Median age , (range) years	48.2 (19-75)	52.7 (32-72)	51.0 (28-72)
Gender (M/F)	26 M / 26 F	9 M / 6 F	27 M / 20 F
Treatment duration (no. patients)			
-medium-term*	-	7 (46.7%)	21 (44.6%)
-long-term†		8 (53.3%)	26 (55.4%)
Dose of medication (no. patients)			
H ₂ RAs			
150 mg daily			-
300 mg daily	-	3 (20%)	
>300 mg daily		10 (69%)	
PPIs		2 (11%)	
20 mg daily	-		32 (68%)
40 mg daily		-	13 (27%)
>40 mg daily			2 (5%)
Endoscopic findings			
GORD ± Barrett oesophagus	-	6 (40%)	20 (42.6%)
Erosive gastritis	11 (21%)	5 (33.3%)	13 (27.6%)
Normal findings	41 (79%)	4 (26.7%)	14 (29.8%)

*6 weeks-1 year; †1 year-8 years.

Fasting intragastric pH in relation to basal serum gastrin and chromogranin A

The degree of gastric acid inhibition during endoscopy, as measured by the fasting intragastric pH, was higher in patients on PPI therapy compared to those on H₂RA therapy or controls and did not differ between patients treated with H₂RAs and controls (**Table 3.2**). Moreover, the subset of patients on long-term therapy with PPIs (n=26) showed stronger suppression of gastric acid secretion at the time of endoscopy than the patients on medium-term PPI therapy (n=21) (**Table 3.3**). No such difference was found between patients on medium- vs long-term H₂RAs.

Basal gastric juice pH values positively correlated with fasting serum gastrin ($r=0.50$; $P=0.0001$) and chromogranin A levels ($r=0.37$; $P=0.0001$).

Table 3.2. Basal serum gastrin, chromogranin A, and fasting intragastric pH in relation to the regimen of medical acid inhibition.

Characteristics	Control group (n=52)		H2RA group (n=15)		PPI group (n=47)	
Fasting intragastric pH	1.75	(1.5-3.5)	2.5	(1.5-4)	5.5	(4-6.5) ^a
Serum gastrin (ng/l)	23.5	(14.3-34.8)	30	(17-35)	79	(45-160) ^b
Serum CgA (nmol/l)	1.8	(1.5-2.2)	2	(1.9-2.3)	2.8	(2-5.9) ^c

Values are expressed as the median and interquartile ranges; Mann-Whitney U test.

^aP<0.0003 vs H₂RAs and P<0.0001 vs controls

^bP<0.001 vs H₂RAs and P<0.0001 vs controls

^cP<0.002 vs H₂RAs and P<0.0001 vs controls.

Table 3.3. Basal serum gastrin, chromogranin A, and fasting intragastric pH in relation to the duration of PPI therapy.

Characteristics	Medium-term PPIs (6 weeks - 1 y)	Long-term PPIs (1 y - 8 y)	P-value
Number of patients	21	26	-
Median age (range) (y)	47 (27-72)	54 (30-70)	NS
Gender (M/F)	13 M / 8F	14 M / 12 F	NS
Fasting intragastric pH	4 (1.5-6.2)	6 (4-6)	<0.03
Serum gastrin (ng/l)	49 (29-78.5)	127 (73-217)	<0.0001
Serum CgA (nmol/l)	2.1 (1.9-2.6)	4.8 (2.8-8)	<0.001

Values are expressed as the median and interquartile ranges; Mann-Whitney U test.

Serum gastrin and CgA measurements

Serum CgA levels positively correlated with serum gastrin in the entire study population ($r=0.55$, $P=0.0001$). An even stronger correlation ($r=0.76$, $P=0.0001$) between serum gastrin and CgA was found in the PPI group (**Figure 3.1**).

Basal serum gastrin and CgA levels were higher in patients taking PPI therapy than in those on H₂RAs or controls but did not differ between patients on H₂RAs and controls (**Table 3.2**). Serum gastrin and CgA levels showed a slight and significant increase with the duration of continuous PPI therapy (**Figure 3.2a, 3.2b**). Likewise, patients on long-term treatment with PPIs (1 to 8 years) had significantly higher serum gastrin and CgA levels compared to those on medium-term (6 weeks to 1 year) PPI therapy (**Table 3.3**). However, there was no difference with respect to serum gastrin and CgA between patients on medium- vs long-term therapy with H₂RAs [30 (15-56) ng/l vs 28 (17-35) ng/l, $P=0.95$ and 2 (1.9-2.3) nmol/l vs 2 (1.8-2.3) nmol/l, $P=0.78$].

Serum CgA levels higher than the reference range were found in 15 of 26 (58%) patients on long-term PPI treatment, 3 of 21 (14%) patients on medium-term PPIs, in none of the patients on H₂RAs and in 1 of the 52 (1.9%) controls. All patients with CgA concentrations above the

reference range had also serum gastrin levels higher than the upper limit of the normal range.

Figure 3.1. Correlation between fasting serum gastrin (ng/l) and chromogranin A (nmol/l) in 47 patients on PPI treatment ($r = 0.76$, $P = 0.0001$). Note: the individual data are plotted on a logarithmic scale.

Basal serum gastrin and chromogranin A in relation with *H.pylori* status

Among the 114 consecutive dyspeptic patients included in the study, 50 (43.8%) were *H.pylori* positive and 64 (56.2%) were *H.pylori* negative. Twenty-four (46%) of the controls, 6 (40%) of the patients on H_2 RAs, and 20 (43%) of the patients on PPIs had positive *H.pylori* serology.

Patients with positive *H.pylori* serology had higher median fasting serum gastrin and median chromogranin A levels compared to patients with negative *H.pylori* serology [51 (27-119) ng/l vs 27 (14-79) ng/l, $P=0.011$; 2.4 (1.9-3.4) nmol/l vs 2.0 (1.7-2.5) nmol/l, $P=0.05$] (**Figure 3.3a, 3.3b**).

Figure 3.2a. Correlation between fasting serum gastrin levels (ng/l) and the duration of PPI treatment (years) in 47 patients ($r = 0.66$, $P = 0.0001$, $y = 51.7 + 36x$). Note: the individual data are plotted on a logarithmic scale.

Figure 3.2b. Correlation between fasting serum chromogranin A levels (nmol/l) and the duration of PPI treatment (years) in 47 patients ($r = 0.41$, $P = 0.005$, $y = 2.75 + 1.74x$). Note: the individual data are plotted on a logarithmic scale.

Discussion

Long-term therapy with potent antiseecretory drugs results in functional and proliferative changes of gastric enterochromaffin-like cells⁴⁶.

Although in humans an achlorhydria-carcinoid sequence seems unlikely, the effect of long-term hypergastrinaemia on the endocrine cells of the gastric oxyntic mucosa remains unclear.

Figure 3.3a. Fasting serum gastrin levels were higher in *H.pylori* positive (Western blotting) than in *H.pylori* negative patients ($P = 0.011$). Median values (interquartile ranges) are shown.

Figure 3.3b. Fasting serum chromogranin A levels were higher in *H.pylori* positive (Western blotting) than in *H.pylori* negative patients ($P = 0.05$). Median values (interquartile ranges) are shown.

The recently described role of ECL cells in the modulation of gastric acid secretion sheds a new light on the pathophysiology of this complex cell population. The ECL cells are thought to increase gastric acid secretion by releasing histamine in response to gastrin stimulation⁴⁷⁻⁴⁹. Hence, prolonged hypergastrinaemia might play a central role in the rebound acid hypersecretion after long-term acid inhibition⁴².

The study of gastric endocrine cells based on morphological methods provides unique information about the progression of the hyperplastic changes. However, the accurate evaluation of gastric mucosal endocrine proliferation encounters many limitations in daily clinical practice. Mucosal ECL cell hyperplasia is not endoscopically detectable and, thus, may be under-estimated in routine examinations⁵⁰. The coexistence of parietal cell hypertrophy / hyperplasia due to hypergastrinaemic states⁵¹, or the presence of *H.pylori*-related atrophic gastritis with clustering of the endocrine cells are possible confounding factors in the assessment of endocrine cell hyperplasia. Moreover, the use of laborious morphometric methodologies with large variations in expressing the results makes a comparison between different studies difficult.

For these reasons, the search for rapid, reliable, and cost-effective strategies to monitor early changes in gastric ECL cell function and growth related to drug-induced hypergastrinaemia is justified.

The present study describes, for the first time, a correlation between serum gastrin and CgA levels during medium- and long-term acid suppressive therapy, suggesting that serum CgA might reflect functional and / or proliferative changes of the gastric ECL cells. This observation is consistent with our previous study involving healthy volunteers and short-term antisecretory therapy with H₂RAs and PPIs, in which the increase in serum gastrin levels following different degrees of pharmacological acid inhibition was accompanied by similar increase in serum CgA levels⁴³. These findings also confirm the results of Waldum *et al.*⁴², who described augmented serum CgA values after short-term treatment with omeprazole and suggested its use as a test to evaluate ECL cell hyperplasia secondary to hypergastrinaemia, during long-term PPI therapy.

Based on a large sample of consecutive dyspeptic patients free of any conditions known to be associated with elevated serum gastrin and CgA levels, we found increased serum gastrin and CgA in patients on PPI therapy, but not in those on H₂RAs, or in nontreated dyspeptic controls with a similar distribution of age and gender. More than 38% of the patients receiving continuous acid inhibition with PPIs (15 of the 26 patients on long-term PPI and 3 of the 21 patients on medium-term PPI therapy) had serum CgA values above the normal range, whereas no

patients on H₂RAs and only one control subject showed such increased levels.

This patient was a 63-year-old man, with a three year-history of persistent epigastric pain and nausea, without weight loss. His past history did not show any relevant events and an abdominal ultrasonography performed prior to endoscopy was normal. Gastroscopy showed no abnormalities. Fasting intragastric pH was 1.5, serum gastrin and CgA were 11 ng/l and 8.2 nmol/l, respectively, and *H.pylori* serology was negative. Routine biochemistry including liver and renal functional tests were normal. The family history revealed no findings suggestive of MEN I. As increased serum CgA levels are not totally specific to neuroendocrine tumours, a subclinical malignant process with neuroendocrine differentiation could account for the high circulating CgA. However, neither before nor after the endoscopy was such condition detected.

In agreement with previous studies, fasting serum gastrin levels in the 47 patients on continuous PPI treatment were related to the degree and duration of gastric acid inhibition^{20,22,29,33,52,53}. Moreover, we found that serum CgA described similar time-dependent changes. Linear regression analysis showed a slight but significant increase in serum CgA levels with the duration of continuous PPI therapy. In spite of similar distribution of the demographic data, patients on long-term (1 to 8 years) treatment with PPI, had significantly higher serum CgA than those who received medium-term (6 weeks to 1 year) PPI therapy.

In a comprehensive study regarding the long-term effects of lansoprazole on serum gastrin, the progression of fundic gastritis and changes in fundic argyrophil cell morphology, Eissele *et al.* found a significant increase in fasting serum gastrin levels within the first 3 months of treatment, with no further elevation in the following five years of therapy²⁹. Despite the plateau-phenomenon described for serum gastrin, argyrophil cell density showed a slight but steady increase during the five-year of follow-up investigation. In our study, even slightly elevated median serum gastrin levels (127 ng/ml) in patients on long-term PPI treatment corresponded to an increase in serum CgA levels. The duration of continuous stimulation rather than the magnitude of hypergastrinaemia seems to be responsible for the elevated serum CgA values in these patients. Whether increased serum CgA reflects only enhanced secretory function of the gastric ECL cells, as an early consequence of sustained hypergastrinaemia, or a late phenomenon, such as ECL cell proliferation, remains to be established.

The results of the current study support the role of *H.pylori* infection in the modulation of gastric ECL cell function and / or growth. Patients with positive *H.pylori* serology showed significantly higher serum gastrin and CgA levels compared to patients with negative *H.pylori* serology.

The forthcoming hypothesis could be that serum CgA might provide additional information with regard to gastric mucosal endocrine and nonendocrine changes in *H.pylori* infected patients on antisecretory therapy.

We realise that the present study may suffer from several confounding factors. The cross-sectional design does not allow conclusions to be drawn concerning the precise *time-course* of serum CgA changes in patients on long-term PPI therapy. However, the significant increase in CgA with the longer period of acid inhibition is a genuine finding. An interesting question emerging in this context is whether persistent increase in serum CgA levels might interfere with gastric acid secretion. The functional role of circulating chromogranins in humans is poorly defined⁵⁴. However, in experimental models, pancreastatin - the main CgA-derived peptide - has been regarded as a potent inhibitor of gastric parietal cell secretion⁵⁵. In view of this, one might speculate that elevated serum CgA, subsequent to drug-induced hypergastrinaemia, may further upregulate gastrin secretion, thus explaining their parallel changes during long-term acid inhibition. On the other hand, reduction of the parietal cell secretion could further potentiate the pharmacologically-induced acid inhibition, in a self-perpetuating process.

In conclusion, we report significantly higher serum gastrin and CgA levels after long-term PPI therapy compared to H₂RA therapy and nontreated dyspeptic controls. A combined effect of *H.pylori* infection and antisecretory therapy on gastric endocrine cells, reflected in increased serum gastrin and CgA levels, is also suggested. A possible relationship between elevated CgA in serum and the presence and severity of gastric mucosal endocrine proliferation remains to be further evaluated.

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Chapter 4

Serum chromogranin A as a screening test for gastric enterochromaffin-like cell hyperplasia during acid-suppressive therapy

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Abstract

Background

Serum chromogranin A (CgA), a marker of neuroendocrine neoplasia, increases during profound gastric acid inhibition, possibly reflecting the trophic effect of gastrin on the enterochromaffin-like (ECL) cells.

Aims

This study investigated the clinical value of serum CgA as a screening test for gastric fundic enterochromaffin-like (ECL) cell hyperplasia during acid-suppressive therapy.

Methods

A consecutive series of 230 dyspeptic patients referred for upper gastrointestinal endoscopy was investigated in a cross-sectional design. They were 154 patients on continuous medium-term (6 weeks to one year) or long-term (longer than one year) acid inhibition with either proton pump inhibitors (PPIs, $n=117$) or histamine₂-receptor antagonists (H₂RAs, $n=37$) for gastro-oesophageal reflux disease, and 76 non-treated subjects, with normal endoscopic findings (control group). Fasting blood samples were analysed for gastrin and CgA. Gastric biopsy specimens (oxyntic mucosa) were examined for histological evaluation of gastritis (Sydney classification) and of ECL cell hyperplasia (Solcia classification).

Results

Serum CgA levels correlated positively with serum gastrin, following a quadratic function ($r=0.78$, $P<0.0001$). Elevated serum CgA values during long-term acid inhibition correlated with the presence and severity of fundic ECL cell hyperplasia. Multivariate analysis identified hypergastrinaemia ($P<0.0001$), duration of acid inhibition ($P<0.0001$), *H.pylori* infection ($P=0.008$), ECL cell hyperplasia ($P=0.012$), and body gland atrophy ($P=0.043$) as independent predictors of elevated serum CgA. In subjects on long-term acid inhibition ($n=123$), serum CgA was equally sensitive but more specific than serum gastrin for the detection of ECL cell hyperplasia (sensitivity, 91.3% for both; specificity, 73% vs 43%, $P<0.0001$).

Conclusions

During long-term gastric acid inhibition, serum CgA levels reflect the presence and severity of fundic ECL cell hyperplasia. Serum CgA is therefore a useful screening test for gastric ECL cell proliferative changes within this context.

Introduction

Gastrin release from the antral G cells of the stomach is negatively regulated by luminal acid and therefore, hypergastrinaemia frequently occurs in association with acid-suppressive therapy¹⁻³. Gastrin has a specific effect on the enterochromaffin-like (ECL) cells of the oxyntic mucosa, stimulating their function and proliferation^{4,5}. Early toxicology studies demonstrated that rats treated long-term with histamine₂-receptor antagonists (H₂RAs)^{2,6} or with proton pump inhibitors (PPIs)^{2,7} develop gastric ECL cell hyperplasia, and subsequently carcinoid tumours.

In humans, this sequence of events has not sufficiently been established, possibly due to the limited follow-up duration of the available studies^{8,9}. Most human gastric ECL cell carcinoids arise on a background of type A chronic atrophic gastritis or in patients with the Zollinger-Ellison syndrome associated with multiple endocrine neoplasia, indicating that hypergastrinaemia, immunological factors and genetic events may act in concert during gastric endocrine tumourigenesis^{10,11}.

It should be pointed out, however, that in patients with pernicious anaemia, massive hypergastrinaemia, lasting more than one decade precedes the development of gastric carcinoids^{12,13}. As endocrine tumour formation may be a very slow process it seems premature to discard the possibility of developing gastric ECL cell carcinoids in response to iatrogenic hypergastrinaemia.

In recent years, the combined effects of antisecretory therapy and *Helicobacter pylori* (*H.pylori*) infection on serum gastrin and gastric endocrine cells have elicited substantial clinical and research interest^{14,15}. Some studies have shown that, *H.pylori* infection - in patients treated with acid suppression - is a risk factor for the progression of corpus (atrophic) gastritis and the development of ECL cell hyperplasia^{15,16}. Undoubtedly, the histological examination is essential for the accurate evaluation of such gastric mucosal changes. Nevertheless, it should be recalled that, the assessment of gastric endocrine proliferation in daily practice is hampered by the fact that such lesions are usually not endoscopically detectable and, thus, may be overlooked. Additionally, the co-existence of chronic atrophic gastritis with 'clustering' of the endocrine cells, or the presence of parietal cell hypertrophy/ hyperplasia secondary to hypergastrinaemia may bias the histological evaluation of ECL cell hyperplasia. To this end, efficient, non-invasive, and unexpensive screening methods to monitor the gastric endocrine lesions in this context are warranted.

Chromogranin A (CgA) - a well-recognised marker of neuroendocrine neoplasia^{17,18} - may be released into the circulation from the ECL cell population of the stomach¹⁹⁻²¹. We and other investigators have reported elevated serum CgA in patients treated short-term^{22,23} and long-term²⁴ with profound gastric acid inhibition. So far, it has not been elucidated whether the increased serum CgA levels under these circumstances only express an *enhanced secretory function* or reflect gastric *ECL cell proliferation*, secondary to drug-induced hypergastrinaemia.

The present study explored the clinico-pathological determinants of serum CgA levels in patients treated with acid suppression and in non-treated controls, with special reference to the role of the *Helicobacter pylori* infection. The issue addressed was whether serum CgA during long-term acid inhibition is a useful screening test for the detection of fundic ECL cell hyperplasia. The utility of serum gastrin, in this respect, was also assessed for comparison.

Patients and methods

Study population

A total of 230 consecutive dyspeptic patients referred for upper gastrointestinal endoscopy to an open-access unit were investigated, in a cross-sectional design. The study group consisted of 154 patients on continuous medium- or long-term acid-suppressive therapy for gastro-oesophageal reflux disease (GORD), including erosive and non-erosive oesophagitis and/or Barrett's oesophagus. Continuous acid-suppressive therapy was defined as at least one daily dose of either omeprazole or ranitidine, taken either 6 weeks to one year (medium-term) or longer than one year (long-term), respectively. The control group consisted of 76 dyspeptic patients, with a similar distribution of age and gender, who did not receive any acid-suppressive medication or antacids before referral, and who had normal endoscopic findings. The following exclusion criteria were applied in all patients: (1) present or past history of peptic ulcer disease; (2) previous gastric surgery and/or vagotomy; (3) prior *H.pylori* eradication therapy; (4) treatment with antimicrobial agents, prokinetic drugs or nonsteroidal anti-inflammatory drugs within 30 days before endoscopy; (5) pancreas or other gastrointestinal pathology, (6) chronic liver or renal diseases; (7) malignancies or endocrine diseases. Subjects gave informed consent prior to inclusion, and the study was approved by the Ethical Review Board of the University Hospital Maastricht, The Netherlands.

Design of the study

General clinical records and a structured interview concerning the history of reflux symptoms, type, daily dose, and duration of treatment with acid-suppressive medication, any comorbidity/ comedication, and family history of gastric cancer were obtained from all patients.

Endoscopy was performed after an overnight fast with standard gastroscopes (Pentax EG-2901). During endoscopy two antral biopsies (2 cm proximal to the pylorus) and four body mucosal biopsies (10 cm below the gastro-oesophageal junction, along the greater curvature) were sampled for histological examination. Additional antral and/or body mucosal biopsies were taken for culture of *H.pylori*.

Fasting blood samples were drawn between 9.00 am and 11.00 am for the determination of serum CgA and serum gastrin levels.

Laboratory assessments

The serum CgA concentration was measured by radioimmunoassay, using polyclonal antibodies raised in rabbits against a fragment of chromogranin A (CgA 116-439) purified from the urine of a patient with a carcinoid tumour^{18,25}. These antibodies bind to both intact and partly degraded CgA. As previously described, this method measures CgA fragments released into the circulation from the ECL cell population of the stomach^{21,26}. This assay has a high precision with intra- and inter-assay coefficients of variation of less than 6.4%²⁵. The serum gastrin concentration was assessed by radioimmunoassay, using antibodies raised in rabbits against synthetic unsulphated human gastrin 2-17 covalently coupled to bovine serum albumin, as described elsewhere²⁷. These antibodies bind to all major forms of circulating gastrin with an almost equal potency. The intra- and inter-assay coefficients of variation with this method are less than 10%. For fasting serum CgA, the upper limit of the normal range was 4 nmol/L¹⁸ and for fasting serum gastrin was 85 ng/L²⁷.

Histopathology

Biopsy specimens for histopathological examination were formalin fixed and paraffin embedded. Serial sections (4 µm) were cut and stained with haematoxylin-eosin for general histological analysis. All biopsy specimens were reviewed by one experienced gastroenterological pathologist (A.B.), without knowledge of the patients' clinical and serological data. Discordant results, although rare, were discussed with a second pathologist and a joint diagnosis was made.

The histomorphologic status of the gastric body mucosa was assessed according to the Sydney classification²⁸. The following parameters were evaluated: active inflammation (*i.e.*, neutrophilic cell

infiltration); chronic inflammation (*i.e.*, mononuclear cell infiltration), and gland atrophy (*i.e.*, loss of appropriate glands). All items were graded from 0 to 3 (0, absent; 1, mild; 2, moderate; 3, severe changes). For analysis of each parameter, the highest grade among the biopsies of the respective gastric area was considered.

In addition, specimens of body mucosa were immunostained for endocrine cells using a monoclonal antibody against human chromogranin A (1:50000) as primary antibody (LK2H10, Hybritech, San Diego, CA, USA)²⁹. This technique stains several types of gastric endocrine cells, such as ECL cells, enterochromaffin cells and A-like (or X) cells, but not the somatostatin-producing D cells. Furthermore, it is known that the ECL cells constitute the predominant endocrine cell type of the gastric oxyntic mucosa (30-50% in humans) and the only one which develop hyperplasia in response to iatrogenic hypergastrinaemia^{3,30}. Therefore, chromogranin A immunostaining ranks as a reliable method to evaluate ECL cell hyperplasia.

The status of the ECL cell population of the corpus mucosa was evaluated according to the Solcia classification³¹. Based on a semi-quantitative histological approach, endocrine hyperplastic lesions have been defined and graded, as follows: 0, absent; 1, diffuse hyperplasia (increase of the number of endocrine cells with coalescence of a few cells); 2, linear hyperplasia (occurrence of at least two linear sequences of five or more endocrine cells per linear millimeter of mucosa); and 3, micronodular hyperplasia (occurrence of at least 1-2 clusters composed of five or more endocrine cells per linear millimeter of mucosa).

Helicobacter pylori

Determination of current *H.pylori* status was performed in both antral and corpus mucosa by modified Giemsa stain, immunohistochemistry (IMM), and culture. For the IMM, a purified polyclonal antiserum (DAKO B471, ITK diagnostics BV, Uithoorn, The Netherlands) in a dilution of 1:100 was used³². Culture of gastric biopsies was performed on selective Campylobacter plates, under microaerophilic conditions. *H.pylori* was considered present if histopathology and/or culture were positive.

Statistical evaluation

Differences in dichotomous variables were evaluated using the chi-square test or Fisher's exact test, depending on the number of patients. As the continuous variables were not normally distributed, values were expressed as medians, with interquartile ranges, and non-parametric tests were used (Kruskal-Wallis test for multiple comparisons and Mann-Whitney *U* test for two group comparisons). Correlations were

performed with the Spearman rank test. Multivariate linear regression analyses with the logarithm of serum CgA as the dependent variable were performed to identify independent predictors of serum CgA levels among the study population. Two-sided P-values <0.05 were assumed to indicate statistical significance.

Results

Study population

The study population comprised 230 patients with complete clinical, serological and histological data. **Table 4.1** summarises the demographic and relevant clinical characteristics of the patients, classified according to the type of acid-suppressive therapy they were receiving. The age distribution and the sex ratios were not statistically different among the three patient groups.

Table 4.1. Demographic, clinical, and endoscopic characteristics of the 230 patients

Characteristics	Controls	H2RA group	PPI group
Number of patients	76	37	117
Age (years)*	51.5(19-79)	52(20-73)	51(21-78)
Male:female ratio	1.05:1	0.95:1	1.3:1
Duration of acid inhibition (months)*	-	32(1.5-216)	29(1.5-168)
Dose of medication (mg daily)*	-	300(150-450)†	40(20-80)‡
Endoscopic findings (%)			
- GORD			
non-erosive	-	62.1	53.8
erosive	-	32.4	16.3
- Barrett's oesophagus	-	5.5	29.9
- normal findings	100	-	-

*Data are expressed as median (range). Daily doses of †ranitidine and ‡omeprazole.

Serum gastrin and chromogranin A in relation to acid inhibition

Serum gastrin levels were higher in the PPI-treated group compared to the H₂RA-treated group and controls (107 [12-800] ng/L vs 51 [8-150] ng/L and 107 [12-800] ng/L vs 30 [5-194] ng/L, P<0.0001 for both). Similarly, serum CgA levels were higher in the PPI group than in the H₂RA group and in controls (3.7 [0.7-26.1] nmol/L vs 2.0 [1.5-5.6] nmol/L and 3.7 [0.7-26.1] nmol/L vs 1.9 [1.2-3.9] nmol/L, P<0.0001 for both). No significant differences with regard to serum gastrin and serum CgA values were found between the H₂RA group and controls. Serum gastrin levels exceeded the upper limit of normal in 78 (66.7%) patients on PPIs, in 9 (24.3%) patients on H₂RAs, and in 8 (10.5%) controls,

while the corresponding figures for serum CgA were 51 (43.6%), 3 (8.1%), and 0 (0%) of the subjects, respectively.

In the total study population fasting serum gastrin correlated positively with fasting serum CgA levels ($r=0.78$, $P<0.0001$) (**Figure 4.1**). This correlation could be fitted best by a quadratic function: $y = 0.159 x^2 - 0.817 x + 1.585$, in which $y = \log(\text{CgA})$ and $x = \log(\text{gastrin})$.

Figure 4.1 The correlation between fasting serum gastrin and fasting serum CgA levels ($r=0.78$; $P<0.0001$; $n=230$) was fitted best by a quadratic function: $y = 0.159 x^2 - 0.817 x + 1.585$, in which $y = \log(\text{CgA})$ and $x = \log(\text{gastrin})$. The different symbols represent the three patient groups: controls (○), H₂RA group (▲), and PPI group (●). The individual data are plotted on a logarithmic scale to accommodate for extreme values.

Serum gastrin, chromogranin A and *H.pylori* infection

H.pylori infection was detected in 32 out of 76 (42.2%) controls, in 15 out of 37 (40.5%) patients in the H₂RA group, and in 43 out of 117 (36.7%) patients in the PPI group.

As shown in **Figures 4.2a** and **4.2b**, *H.pylori*-positive subjects had higher serum gastrin and higher serum CgA levels than the *H. pylori*-negative subjects, across all three patient groups. The highest serum gastrin and CgA values were observed in the *H.pylori*-positive patients treated with PPIs.

Figure 4.2 Fasting serum gastrin (a) and fasting serum CgA (b) levels in *H. pylori*-negative (O) and *H. pylori*-positive (●) subjects. The shaded area indicates the normal range. The horizontal bars indicate median values. The individual data are plotted on a logarithmic scale to accommodate for extreme values. Numbers of subjects in parentheses.

Serum gastrin, chromogranin A and body gastritis

Table 4.2 describes the parameters of body gastritis in all patients. As expected, *H.pylori*-positive subjects yielded a higher prevalence of active inflammation, chronic inflammation and body gland atrophy than the *H.pylori*-negative subjects, across all three patient groups. The highest prevalence and severity of body gland atrophy were observed in the *H.pylori*-positive patients treated with PPIs.

In the study population, patients with body gland atrophy had significantly higher serum gastrin and serum CgA levels than those without it (serum gastrin: 114.5 [25-800] ng/L vs 58 [5-253] ng/L, $P=0.008$ and serum CgA: 3.6 [1.1-26.1] nmol/L vs 2.3 [1.2-14.4] nmol/L, $P=0.004$).

Serum gastrin, chromogranin A and fundic ECL cell hyperplasia

As shown in **Table 4.2**, the prevalence of ECL cell hyperplasia was higher in the PPI group than in the H₂RA group (18.8% vs 5.4%, $P=0.05$) and in the controls (18.8% vs 3.9%, $P<0.004$), whereas no significant difference in this respect was found between patients on H₂RAs and controls. Additionally, in the entire series, *H.pylori*-positive subjects had a higher prevalence of ECL cell hyperplasia than the *H.pylori*-negative subjects (23.4% vs 4.3%, $P<0.0001$).

As illustrated in **Figures 4.3a** and **4.3b**, the patients with ECL cell hyperplasia - either *H.pylori*-negative or -positive - had higher serum gastrin and higher serum CgA levels than those without ECL cell hyperplasia. Moreover, serum gastrin and serum CgA correlated positively with the histological degree of ECL cell hyperplasia (**Figures 4.4a** and **4.4b**). The group of *H.pylori*-positive patients treated with PPIs had the highest prevalence and severity of fundic ECL cell hyperplasia (**Table 4.2**). None of the specimens investigated showed either adenomatoid, dysplastic lesions, or carcinoids.

Table 4.2. Prevalence and severity of active inflammation, chronic inflammation, gland atrophy, and ECL cell hyperplasia of the body mucosa in *H.pylori*-negative and *H.pylori*-positive patients on acid-suppressive therapy and in non-treated controls

Corpus gastritis	Controls		H2RA group		PPI group	
	<i>H.pylori</i> (-) (n=44)	<i>H.pylori</i> (+) (n=32)	<i>H.pylori</i> (-) (n=22)	<i>H.pylori</i> (+) (n=15)	<i>H.pylori</i> (-) (n=74)	<i>H.pylori</i> (+) (n=43)
Active inflammation	2 (4.6%)	22 (68.7%)	0 (0%)	13 (86.7%)	4 (5.4%)	35 (81.4%)
Grade 1,2,3*	1;0;1	13;4;5	0;0;0	8;4;1	2;2;0	24;10;1
Chronic inflammation	2 (4.6%)	24 (74%)	1 (4.5%)	9 (60%)	4 (5.5%)	42 (97.7%)
Grade 1,2,3*	1;1;0	18;4;2	1;0;0	6;2;1	1;2;1	22;12;8
Gland atrophy	1 (2.3%)	8 (25%)	1 (4.5%)	7 (46.7%)	2 (2.7%)	23 (53.5%)
Grade 1,2,3*	1;0;0	4;2;2	1;0;0	3;2;2	2;0;0	9;8;6
ECL cell hyperplasia	2 (4.5%)	1 (3.1%)	0 (0%)	2 (13.4%)	4 (5.4%)	18 (41.9%)
Grade 1,2,3*	2;0;0	1;0;0	0;0;0	1;1;0	3;1;0	15;1;2

* Number of subjects with different severity of active inflammation, chronic inflammation, body gland atrophy (eg, 1;0;1 indicates 1 subject with grade 1 [mild], 0 with grade 2 [moderate], and 1 with grade 3 [severe]), and ECL cell hyperplasia (eg, 2;0;0 indicates 2 subjects with grade 1 [diffuse type], 0 with grade 2 [linear type], and 0 with grade 3 [micronodular type]).

Figure 4.3 Fasting serum gastrin (a) and fasting serum CgA (b) levels in relation to ECL cell hyperplasia and *H.pylori* infection. The 50th percentile (median) is indicated with a line in the box, the end of the box indicates the 25th and 75th percentile, and the 10th and 90th percentile are indicated with error bars. Numbers of subjects in parentheses.

Figure 4.4 Fasting serum gastrin (a) and fasting serum CgA (b) levels in relation to the histological degree of ECL cell hyperplasia. The 50th percentile (median) is indicated with a line in the box, the end of the box indicates the 25th and 75th percentile, and the 10th and 90th percentile are indicated with error bars. Numbers of subjects in parentheses.

Figure 4.5 shows the relationship between serum gastrin, serum CgA, gastric fundic ECL cell hyperplasia, and the duration of acid-suppressive therapy. The total study population was subdivided into six subgroups, according to the duration of treatment they had undergone. No significant differences with regard to age, gender and prevalence of *H.pylori* infection were observed among the six patient subgroups ($P=0.76$, $P=0.95$, $P=0.74$, respectively). High serum gastrin levels ($>85\text{ng/L}$) were present in a large proportion of patients, with a nearly stable rate after one year of treatment. However, the frequency of elevated serum CgA levels ($>4\text{nmol/L}$) increased after acid suppression longer than one year, and was paralleled by a gradual increase in the prevalence of ECL cell hyperplasia (3.2% of patients treated <12 months vs 33.4% of patients treated >48 months, $P=0.003$).

* $P<0.003$, $†P<0.001$ and $‡P=0.15$ vs patients treated <12 months.

Figure 4.5. Relationship between serum gastrin, serum CgA levels, ECL cell hyperplasia, and the duration of acid-suppressive therapy.

Table 4.3 shows the clinico-pathological characteristics of the five patients with more severe (linear or micronodular) pattern of ECL cell hyperplasia.

Table 4.3. Performance characteristics of serum CgA and serum gastrin for the detection of ECL cell hyperplasia in 123 patients treated long-term (>1 year) with acid-suppressive therapy

No	Age (years)	Gender	Type, dose, duration of therapy	<i>Hp</i> status	Body gastritis*	ECL cell hyperplasia	Serum gastrin (ng/L)	Serum CgA (nmol/L)
1	49	F	ome 20-40 mg/day 48 months	pos	1;2;1	linear	205	17.1
130	71	F	ome 40-80 mg/day 36 months	neg	1;2;3	linear	400	15
156	78	M	rani 300-450 mg/day 96 months	pos	0;2;3	linear	500	17.3
197	68	F	ome 40 mg/day 56 months	pos	1;3;2	miconodular	800	23
230	72	F	ome 40-80 mg/day 72 months	pos	0;2;2	miconodular	800	25

*histological degree of body gastritis (e.g. 1;2;1 indicates active inflammation grade 1 [mild]; chronic inflammation grade 2 [moderate]; and body gland atrophy grade 1 [mild]).

Multivariate analysis

Multiple stepwise linear regression analysis was performed to identify determinants of serum CgA levels in the study population. Hypergastrinaemia ($P<0.0001$), duration of acid-suppressive therapy in years ($P<0.0001$), infection with *H.pylori* ($P=0.008$), fundic ECL cell hyperplasia ($P=0.012$) and body gland atrophy ($P=0.043$) were independent predictors of elevated serum CgA values. Age and gender did not significantly influence serum CgA levels. No interactions between variables were found.

Clinical utility of serum chromogranin A in screening for ECL cell hyperplasia

As the prevalence of ECL cell hyperplasia progressively increased in patients treated with acid suppression longer than one year ($n=123$), we further examined the clinical utility of serum CgA as a screening test for ECL cell hyperplasia in these patients, by comparing it to serum gastrin in this respect. Among these 123 patients, elevated serum gastrin (>85 ng/L) were observed in 74 (60%) cases, elevated serum CgA (>4 nmol/L) in 48 (39%) cases, and 23 (18.7%) cases displayed histological signs of ECL cell hyperplasia. Twenty-one of the 74 subjects with elevated serum gastrin displayed ECL cell hyperplasia. Likewise, 21 of the 48 subjects with elevated serum CgA displayed ECL cell hyperplasia. The test performance characteristics for serum gastrin and serum CgA in the detection of ECL cell hyperplasia are detailed in **Table 4.4**. The sensitivity of serum CgA was high and equal to that of serum gastrin (91.3%). However, serum CgA had a significantly higher specificity compared to serum gastrin (73% vs 47%, $P<0.0001$). Both parameters showed good negative predictive values for ECL cell hyperplasia (serum CgA: 97.3% and serum gastrin: 95.9%, respectively), but limited positive predictive values (serum CgA: 43.8% and serum gastrin: 28.4%, respectively).

Table 4.4. Performance characteristics of serum CgA and serum gastrin in the detection of ECL cell hyperplasia in 123 patients treated long-term (>1 year) with acid-suppressive therapy.

	Serum chromogranin A*	Serum gastrin*
Sensitivity (%)	91	91
Specificity (%)	73	47
Positive predictive value (%)	44	28
Negative predictive value (%)	97	96

*Normal range for serum CgA was defined as <4 nmol/L and for serum gastrin as <85 ng/L.

Discussion

The current study confirmed that gastric fundic ECL cell hyperplasia develops during long-term profound acid suppression - particularly in *H.pylori*-positive patients - and has shown that this process is well reflected by increased serum CgA levels. This holds true for serum gastrin regarding test sensitivity. However, serum CgA has a better specificity than serum gastrin, being a more reliable test for the detection of ECL cell hyperplasia in patients treated long-term with acid suppression. Multivariate analysis identified hypergastrinaemia, the duration of acid-suppressive therapy, *H.pylori* infection, fundic ECL cell hyperplasia and body gland atrophy as independent predictors of increased serum CgA values. As such, several biological hypotheses may explain the observed elevations in serum CgA during treatment with antisecretory medication.

Acid-related mechanisms play an essential role in this multifactorial process. In a recent study concerning the long-term effects of the proton pump inhibitor lansoprazole on serum gastrin and gastric endocrine mucosa, Eissele *et al.* found a significant increase in fasting serum gastrin levels within the first 3 months of treatment, without further elevation in the following 5 years of therapy¹⁵. This moderate and self-limited hypergastrinaemia was, however, accompanied by a steady increase in the ECL cell density during the 5-year follow-up. These findings in humans reproduce previous reports in rats³³, emphasizing the critical role of the *duration* of stimulation in the proliferation of ECL cells. Our results further substantiate and extend these data by demonstrating the additional value of serum CgA for the assessment of the gastric ECL cell status. As shown in **Figure 4.1**, at a moderate elevation in serum gastrin level - compatible with the known plateau-phenomenon - serum CgA levels increase, following a quadratic function. Interestingly, **Figure 4.5** indicates that in contrast to serum gastrin concentrations - which tend to level off - serum CgA levels gradually rise and are accompanied by an increasing prevalence of ECL cell hyperplasia, affecting one third of the patients after four years of antisecretory treatment. In view of these observations, we consider that increased serum CgA during acid-suppressive therapy initially might reflect a *hypersecretory state* of the ECL cells, as an early response to sustained hypergastrinaemia, but in patients treated long-term with acid suppression such increase is more likely to be the result of *ECL cell hyperplasia*. In line with this, previous data in rats also indicated that, as opposed to low-molecular fragments of CgA (e.g., pancreastatin), which are more influenced by the ECL cell hypersecretory state, the high-molecular forms of CgA reflect more accurately the ECL cell mass^{34,35}.

In this study, elevated serum gastrin and serum CgA, as well as fundic ECL cell hyperplasia were mainly observed in patients treated with PPIs. However, in contrast to earlier reports, that precluded the development of ECL cell proliferative lesions following treatment with H₂RAs³⁶, we found elevated serum CgA and ECL cell hyperplasia also in a small proportion of patients treated with H₂RAs. These results support the contention that fundic ECL cell hyperplasia may develop during long-term acid inhibition with any type of effective antisecretory drug.

This is, to our knowledge, the first study to provide insight into the relationship between *H.pylori* infection, gastric mucosal morphology, and serum CgA levels during antisecretory therapy. We report higher fasting serum gastrin and serum CgA levels in the *H.pylori*-positive compared to the *H.pylori*-negative subjects. Additionally, we suggest that the markedly increased serum gastrin and CgA concentrations in *H.pylori*-positive patients treated with PPIs reflected more advanced body gland atrophy and proliferation of the ECL cells.

Of particular note was the observation that, the magnitude of serum CgA levels correlated positively with the histological degree of ECL cell hyperplasia. In fact, the five patients with either linear or micronodular pattern of ECL cell hyperplasia had serum CgA levels on average five-fold higher than in those with a diffuse pattern. In a study by Borch *et al.* on patients with type A chronic atrophic gastritis, CgA concentrations correlated to endocrine cell densities in the fundic mucosa²¹. The correlation between serum CgA and the histological degree of ECL cell hyperplasia found in our series is in line with such data. The number of patients with an advanced type of endocrine cell growth was, however, small; therefore, this relation needs to be confirmed in further studies.

The results of the current study shed some light on a controversial clinical issue: *Should patients on long-term acid-suppressive therapy be routinely screened for the early detection of gastric ECL cell hyperplasia, in order to prevent carcinoid tumour formation?* Although one single case report⁹ gives some warning, 10 to 15 years of worldwide experience with potent inhibitors of gastric acid secretion does not justify major concern. The question remains, however, still open for the large number of patients considered for lifelong acid suppression, because of gastro-oesophageal reflux diseases that are not referred to surgery. In these patients, it seems advisable to monitor serum CgA levels instead of the less specific serum gastrin. At markedly increased CgA values indication should be given for endoscopic and histological examination of the gastric mucosa.

A few comments have to be made regarding methodological aspects of this study: First, we investigated a large series of consecutive patients, recruited from a primary care population; this confers

generality to the results presented and conclusions. Second, the study was cross-sectional in design; however, the reliable patient histories concerning the length of antisecretory therapy and the comparison with non-treated controls with similar demographic features, enabled us to estimate the role of the time factor in the development of ECL cell hyperplasia. Third, all subjects were free of any conditions known to interfere with serum CgA concentrations^{21,37,38}, thereby supporting the notion that the elevated CgA values during acid-suppressive therapy accurately reflect the stimulation of the gastric ECL cells. Forth, in the present study fasting levels of serum gastrin and serum CgA were measured. The determination of non-fasting values was beyond our scope, but it is also unlikely that meal-stimulated values of serum CgA would provide any additional information to fasting CgA³⁹. It can be expected that at a maximal gastrin stimulation, serum CgA may level off. Consequently, any additional increase in serum gastrin, due to meal-stimulation, would have relatively little effect on serum CgA. This hypothesis is in line with experimental data indicating that pancreastatin - the main CgA-derived peptide - exerts an inhibitory effect on gastric parietal cells⁴⁰ and thereby, may further up-regulate gastrin secretion in a self-perpetuating process.

In conclusion, we have shown that, serum CgA during long-term acid-suppressive therapy, is an equally sensitive and more specific, non-invasive marker of ECL cell hyperplasia than serum gastrin. In view of the potential negative consequences of iatrogenic hypergastrinaemia on the gastric endocrine mucosa, it seems prudent to monitor serum CgA levels in patients treated long-term with profound acid suppression, as they reflect the presence and severity of ECL cell hyperplasia.

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Chapter 5

Non-*Helicobacter pylori* bacterial flora during acid-suppressive therapy: differential findings in gastric juice and gastric mucosa

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Abstract

Background

Intragastric growth of non-*H.pylori* bacteria commonly occurs during acid-suppressive therapy. The long-term clinical consequences are still unclear. The aim of this study was to investigate the *luminal* and *mucosal* bacterial growth during gastric acid inhibition, in relation to the type and duration of acid-inhibitory treatment, as well as to concomitant *H.pylori* infection.

Methods

145 patients on continuous acid inhibition with either proton pump inhibitors (PPIs, n=109) or with histamine-₂ receptor antagonists (H₂RAs, n=36) for gastro-oesophageal reflux disease, and 75 dyspeptic patients without acid inhibition (control group) were included. At endoscopy, fasting gastric juice was obtained for pH measurement and bacteriological culture. Gastric biopsy specimens were examined for detection of *H.pylori* (immunohistochemistry [IMM]) and of non-*H.pylori* bacteria (modified Giemsa stain positive and IMM negative at the same location).

Results

Overall, non-*H.pylori* flora was detected in the gastric juice of 92 (41.8%) patients and in the gastric mucosa of 109 (49.6%) patients. In gastric juice, prevalence rate for non-*H.pylori* bacteria was higher in patients on PPIs than controls and H₂RAs (58.7% vs 22.6% and vs 30.6%, $P<0.0001$ and $P<0.003$, respectively), but did not differ statistically between H₂RAs and controls. In gastric mucosa, prevalence rates for non-*H.pylori* bacteria were higher in patients on PPIs and on H₂RAs than in the controls (antrum: 46.9% and 48.6% vs 25%, $P<0.05$ for both; corpus: 52.2% and 56.8% vs 23.7%, $P<0.001$ for both), but did not differ between PPIs and H₂RAs. Both luminal and mucosal growth of non-*H.pylori* bacteria were significantly greater in *H.pylori*-positive than -negative patients on PPIs ($P<0.05$ for both). Luminal growth of non-*H.pylori* flora increased with the intragastric pH level, while mucosal bacterial growth increased with the duration of acid inhibition.

Conclusions

Non-*H.pylori* flora not only contaminates the gastric juice but also colonises the gastric mucosa of a large proportion of patients treated long-term with acid inhibition. The relation between *H.pylori* and non-*H.pylori* bacteria in the pathogenesis of atrophic gastritis and gastric cancer needs further elucidation.

Introduction

The acidic milieu of the stomach represents the first line of defence against ingested bacteria^{1,2}. An intragastric pH below 4 has a bactericidal effect³, while gastric juice with a pH above 4 enables bacterial colonisation of the stomach^{4,5}.

Decreased gastric acid output may occur as a consequence of *Helicobacter pylori* gastritis⁶ or may be iatrogenically induced by gastric surgery⁷ or administration of acid-suppressive therapy^{8,9}. Earlier studies on intragastric bacterial overgrowth due to acid inhibitory medication brought into focus possible adverse events such as episodes of gastroenteritis¹⁰, development of malabsorption syndromes¹¹, and formation of potentially carcinogenic N-nitroso compounds¹²⁻¹⁶.

In recent years, considerable interest has emerged for the interactions between *H.pylori*, non-*H.pylori* bacteria, and acid-suppressive therapy. Some investigators have shown that, in *H.pylori*-positive patients, long-term acid suppression leads to a more rapid development of atrophic corpus gastritis, a condition associated with an elevated risk of gastric cancer¹⁷⁻²¹. Previous work of our group has indicated that this might be due to a co-infection of the stomach with non-*H.pylori* bacterial flora - secondary to acid inhibition^{22,23}. It is therefore important to characterise more fully the determinants of non-*H.pylori* bacterial growth in such conditions.

In most clinical studies, the intragastric bacterial overgrowth was investigated by culture of gastric juice¹³⁻¹⁶ and only rarely of gastric mucosal tissue²⁴. Recently, our group has proposed a semi-quantitative assessment of the non-*H.pylori* bacteria in gastric mucosa, using a non-specific stain for total bacterial flora combined with a specific immunohistochemical stain for the detection of *H.pylori*^{25,26}.

In the present study we investigated the gastric *luminal* and *mucosal* growth of non-*H.pylori* bacteria, in groups of patients treated with acid inhibition compared to non-treated controls, in order to estimate their potential pathogenetic effect on the gastric mucosa. Special attention was paid to the impact of clinical features, type, degree and duration of acid-suppressive therapy, as well as concomitant *H.pylori* infection on the *luminal* and *mucosal* non-*H.pylori* bacterial growth.

Patients and methods

Study population

A total of 220 consecutive dyspeptic patients referred for upper gastrointestinal endoscopy to an open-access unit were enrolled in a cross-sectional, case-control study. Case subjects were patients on continuous acid-suppressive therapy for gastro-oesophageal reflux diseases (GORD), such as erosive and non-erosive oesophagitis and/or Barrett's oesophagus. Continuous acid suppression was defined as at least one daily dose of either a proton pump inhibitor (PPI) or a histamine₂-receptor antagonist (H₂RA), taken either 6 weeks to one year (medium-term) or longer than one year (long-term), respectively. Eligible controls were dyspeptic patients with a similar distribution of age and gender, who did not receive acid-suppressive medication or antacids before referral and who had normal endoscopic findings. Exclusion criteria for all patients were (1) present or past history of peptic ulcer disease; (2) previous gastric surgery and/or vagotomy; (3) prior *H.pylori* eradication therapy; (4) treatment with antimicrobial agents or prokinetic drugs within 30 days before endoscopy; (5) chronic use of NSAIDs; (6) other gastrointestinal or major non-gastrointestinal pathology, including immune-compromising states. The study was approved by the Ethical Review Board of the University Hospital Maastricht and each subject gave informed oral consent before entering into the study.

Design of the study

General clinical records and a structured interview concerning the history of reflux symptoms, type, daily dose, and duration of acid-suppressive medication, any comorbidity, comedication or family history of gastric cancer were obtained from all patients.

Endoscopy was performed after an overnight fast using a carefully disinfected Pentax EG-2901 instrument. At the beginning of endoscopy, a 5-10 ml sample of gastric juice was obtained - through the suction channel of the endoscope into a sterile trap - for bacteriological culture. Fasting gastric juice pH was measured using pH paper strips with grading steps of 0.5 from pH 0 to pH 14 (Schleicher & Schüll GmbH; Dassel, Germany)²⁷. During endoscopy two antral biopsies (2-3 cm proximal to the pylorus) and four corpus biopsies (midpart of the body mucosa, 4-5 cm above the corpus/antrum junction, along the greater curvature) were sampled for histological examination.

Culture

Samples of gastric juice were transported immediately to the laboratory and were processed within 4 hours. Culture was performed according to

the five segments method, inoculating 1, 10 and 100 μl onto each of the following plates: blood agar plates (Becton Dickinson 254098, Leiden, The Netherlands) for total bacterial counts, cysteine lactose electrolyte-deficient agar plates (C.L.E.D., Becton Dickinson 255529, Leiden, The Netherlands) for Gram-negative rods, anaerobic blood agar plates (Becton Dickinson 254084, Leiden, The Netherlands) for total anaerobic bacterial counts, and selective plates for *Helicobacter pylori*. For the latter species, *Campylobacter* and chocolate agar plates were used, which were incubated under microaerophilic conditions, at 37°C, for four to seven days. All other plates were incubated for a minimum of 48 hours with first reading at 24 hours for aerobes, and at 48 hours for anaerobes. Bacterial growth was assessed semi-quantitatively as colony forming units (CFU) per ml gastric juice, with a lowest detection limit of 10^1CFU/ml and an upper detection limit of 10^7CFU/ml . The degree of bacterial colonisation was scored as follows: grade 0, no bacteria detected; grade 1, $<10^3\text{CFU/ml}$; grade 2, $10^4\text{--}10^6\text{CFU/ml}$ and grade 3, $>10^6\text{CFU/ml}$. Bacteria were identified by standard microbiological methods to species level²⁸.

Histopathology

Serial sections of 4 μm from formalin-fixed and paraffin-embedded specimens were prepared for haematoxylin-eosin stain (H&E), modified Giemsa stain (MG) and an immunohistochemical stain (IMM). IMM with a purified polyclonal antiserum (DAKO B471, ITK diagnostics BV, Uithoorn, The Netherlands) in a dilution of 1:100 was applied to detect *H.pylori*. The presence of non-*H.pylori* bacterial flora was screened in the MG stain, by morphology and location of the bacteria, as described previously^{25,26}. To ascertain the presence of non-*H.pylori* bacteria, the IMM stain of an adjacent section had to be *H.pylori*-negative on the same position. At least two adjacent sections of all biopsies were examined completely at 400x magnification. If necessary a 1000x magnification was used. This technique accurately detects the presence of non-*H.pylori* bacteria when compared to culture methods²⁶.

The presence of both *H.pylori* and non-*H.pylori* bacteria was scored semi-quantitatively and graded as follows: grade 0, no bacteria detected; grade 1, occasionally bacteria detected; grade 2, scattered bacteria identified in several high power fields; grade 3, large amounts detected in many high power fields²⁹. All slides were reviewed by an experienced gastroenterological pathologist (A.B.) and by the main investigator (S.S.), without knowledge of patient identification. In the event of discordant results, the specimens were re-examined by a second pathologist and were discussed until agreement was reached.

Statistical analysis

Differences in dichotomous variables were analysed using unpaired non-parametric tests (chi-square test with Yates correction) or paired non-parametric tests (Wilcoxon's rank-sum test). Differences in continuous variables were analysed using the Kruskal-Wallis test for multiple comparisons and the Mann-Whitney U test for two group comparisons. Two-sided P-values <0.05 were assumed to indicate statistical significance.

Results

Study population

Two-hundred twenty consecutive patients were enrolled in the study. The demographic and relevant clinical features are detailed in **Table 5.1**. Patients were subdivided in three groups, on the basis of the type of acid-suppressive therapy they were receiving: a PPI group, a H₂RA group, and non-treated controls, respectively. The age distribution and sex ratios did not differ statistically among the three patient groups.

Table 5.1. Demographic, clinical and endoscopic characteristics of 220 patients enrolled in the study.

Characteristics	Controls n=75	H ₂ RA group n=36	PPI group n=109
Age (years)*	52 (19-79)	52 (20-72)	51.5 (21-78)
Gender (male:female ratio)	1.03:1	0.9:1	1.3:1
Duration of acid inhibition (months)*	-	32 (1.5-216)	29 (1.5-168)
Dose of medication (mg daily)*	-	300(150-450)†	40(20-80)‡
Endoscopic findings (%)			
- GORD			
• non-erosive	-	61.1	55.9
• erosive	-	33.3	15.0
Barrett's oesophagus	-	5.5	29.1
Normal findings	100	-	-

*Data are expressed as median (range). Daily doses of †ranitidine and of ‡omeprazole.

Prevalence and density of bacterial flora in gastric juice and gastric mucosa

Overall, 56 (25.5%) patients displayed non-*H.pylori* bacteria simultaneously in gastric juice and in gastric mucosa, 36 (16.3%) patients had non-*H.pylori* flora in gastric juice only, 53 (24.1%) in gastric mucosa only, while the remaining 75 (34.1%) patients had no evidence of non-*H.pylori* flora.

Gastric juice

Table 5.2 describes the prevalence and density of non-*H.pylori* bacteria in the gastric juice. Patients on PPIs had higher prevalence of non-*H.pylori* bacteria, particularly oropharyngeal flora compared to the controls ($P<0.0001$) and to the patients on H₂RAs ($P<0.003$), whereas no significant differences were noticed between the H₂RAs and controls. Oropharyngeal flora, including *Neisseria* spp., *Streptococci* spp., and *Corynebacterium* spp. was identified in 16 (21.3%) controls, in 10 (27.8%) patients on H₂RAs and in 64 (58.7%) patients on PPIs. Gram-negative Enterobacteriaceae were recovered from one (1.3%) control subject (*Escherichia coli*), one (2.8%) patient on H₂RAs (*Escherichia coli* and *Proteus mirabilis*) and five (4.6%) patients on PPIs (*Escherichia coli* in three patients, *Proteus mirabilis* in one patient, *Enterobacter gergoviae* in one patient and *Klebsiella oxytoca* in one patient). Anaerobes (bacteroides species) were recovered from two patients on PPIs. In only 1 (1.3%) control subject and 1 (0.9%) patient on PPIs, *H.pylori* bacteria were isolated by culture of gastric juice.

Table 5.2. Prevalence and density of non-*H.pylori* bacterial flora in gastric juice.

	Controls n=75	H ₂ RA group n=36	PPI group n=109
Non-<i>H.pylori</i> flora			
• Oropharyngeal-like (%)	16 (21.3%)	10 (27.8%)	64 (58.7%)^{†,‡}
Grade 1;2;3	3;9;4	2;4;4	2;30;32
• Faecal-like (%)	1 (1.3%)	1 (2.8%)	5 (4.6%)*
Grade 1;2;3	0;1;0	0;1;0	1;2;2
No flora	58 (77.3%)	25 (69.4%)	45 (41.3%)

[†] $P<0.0001$ vs controls and [‡] $P<0.003$ vs H₂RA group.

*all 5 patients had both oropharyngeal-like and faecal-like flora.

Gastric mucosa

Figure 5.1 illustrates the presence and distribution of the *H.pylori* and non-*H.pylori* bacteria in the gastric mucosa of patients on acid inhibition and non-treated controls. The prevalence of *H.pylori* did not differ significantly between the three patient groups. However, the prevalence of non-*H.pylori* flora - in both antrum and corpus - was significantly higher in patients on acid suppression, either with PPIs or with H₂RAs, than controls [antrum: $P=0.003$ and $P=0.021$, respectively; corpus: $P<0.0001$ and $P<0.001$, respectively]. No significant difference in this respect was found between the PPI group and the H₂RA group. The distribution of *H.pylori* between antral and corpus mucosa was similar in patients on H₂RAs and controls, while patients on PPIs had a lower prevalence of *H.pylori* in antral than corpus mucosa (27.4% vs 34.5%,

$P=0.033$). The distribution of non-*H.pylori* flora between antral and corpus mucosa did not differ significantly, within all three groups.

Figure 5.1. Prevalence and distribution of *H.pylori* and of non-*H.pylori* bacteria in gastric mucosa of patients on acid inhibition and non-treated controls.

Figures 5.2a and **5.2b** depict the density of *H.pylori* and non-*H.pylori* bacteria in the antral and corpus mucosa. In the antral mucosa, patients on PPIs displayed lower density (grade 1-3) of *H.pylori* than controls ($P=0.002$), whereas no significant differences were found between patients on H₂RAs and controls, or between patients on PPIs and those on H₂RAs (**Figure 5.2a**). In the corpus mucosa, a similar density of *H.pylori* was found among the three groups. The patients on acid suppression, either with PPIs or with H₂RAs displayed higher density (grade 1-3) of non-*H.pylori* bacteria - in both antrum and corpus mucosa - compared to controls [antrum: $P=0.012$ and $P=0.005$; corpus: $P=0.05$ and $P=0.024$, respectively] (**Figure 5.2b**). No significant difference in the density of non-*H.pylori* flora was seen between the PPI group and the H₂RA group. Also, within each group, no such differences were observed between antral and corpus mucosa.

Histologically, the non-*H.pylori* flora consisted mainly of cocci, although different types of rods were also detected. Most non-*H.pylori* bacteria were localised superficially, in the mucous layer (**Figure 5.3a**), but occasionally were also seen in the glandular lumina, on similar mucosal positions as the *H.pylori* (**Figures 5.3b** and **5.3c**).

Figure 5.2. Density of *H.pylori* (a) and of non-*H.pylori* (b) bacteria in gastric mucosa of patients on acid inhibition and non-treated controls.

Figure 5.3. (A) Massive amounts of cocci present in the gastric mucous layer (1000x objective) from a patient treated long-term with proton pump inhibitors. (B) Presence of cocci and rods in a gastric crypt from a patient with chronic atrophic gastritis and intestinal metaplasia. Bacteria were identified as non-*H.pylori* species by MG (1000x objective) and simultaneous negative IMM. (C) Presence of *H.pylori* in a gastric crypt by IMM (1000x objective).

Intragastric bacterial flora in relation to the type, degree and duration of acid-suppressive therapy

Fasting gastric juice pH values above 4 were measured more frequently in patients on PPIs than in those on H₂RAs and controls (71.6% vs 19.4% and 14.7%, respectively, $P < 0.0001$ for both), whereas no significant differences were noticed between patients on H₂RAs and controls.

H. pylori-positive subjects had significantly higher fasting gastric juice pH than the *H. pylori*-negative, among all three groups (**Figure 5.4**). The highest gastric juice pH levels were observed in *H. pylori*-positive patients treated with PPIs. Also, in patients treated long-term with PPIs, the prevalence of non-*H. pylori* bacteria - in both gastric juice and gastric mucosa - was significantly higher in *H. pylori*-positive than -negative subjects (gastric juice: 26 of 32 [81.2%] vs 32 of 55 [58.2%], $P = 0.028$; gastric mucosa: 27 of 32 [84.3%] vs 33 of 55 [60%], $P = 0.018$, respectively).

Figure 5.4. Median fasting gastric juice pH in *H. pylori* (Hp) negative (□) and in *H. pylori* positive (●) subjects.

The error bars show the 10th and the 90th interquartile ranges.

The patients with non-*H. pylori* bacteria in gastric juice were characterised by higher gastric juice pH level than those without it (**Figure 5.5a**). No such relation was found between the presence of

non-*H.pylori* bacteria in the gastric mucosa and gastric juice pH level (Figure 5.5b).

(a)

(b)

Figure 5.5. Median fasting gastric juice pH in patients without (□) and in those with (●) non-*H.pylori* (nHp) bacteria in *gastric juice* (a) and *gastric mucosa* (b). The error bars show the 10th and the 90th interquartile ranges.

In the whole series five categories of patients were distinguished according to the duration of acid-suppressive therapy they had undergone (**Figures 5.6a** and **5.6b**). Age and gender did not differ significantly among the five categories of patients ($P=0.76$ and $P=0.95$, respectively). In gastric juice, the prevalence of non-*H.pylori* bacteria increased with the duration of acid suppression in patients on PPIs, but not in those on H₂RAs ($P<0.0001$ and $P=0.63$ for patients treated > 36 months vs those treated < 12 months, respectively) (**Figure 5.6a**). In gastric mucosa, the prevalence of non-*H.pylori* bacteria increased with the duration of acid suppression, regardless of the type of antisecretory medication ($P<0.0001$ and $P<0.05$ for patients treated with PPIs and with H₂RAs > 36 months vs < 12 months, respectively) (**Figure 5.6b**).

Discussion

The current study documented a high prevalence and density of the non-*H.pylori* bacterial flora in the gastric mucosa of patients on acid-suppressive therapy. Previous reports have mainly focused on the *luminal* bacterial growth during natural and artificial hypochlorhydria and achlorhydria³⁻⁵. Hitherto, only very few studies provided insights into the *mucosal* bacterial growth²⁴, and none at all investigated the relationship between the two intragastric compartments.

Gastric luminal non-*H.pylori* bacterial growth

In the present study, fasting gastric juice pH levels were higher in *H.pylori*-positive compared to *H.pylori*-negative subjects, especially in the PPI-treated group. Also, *H.pylori*-positive patients on PPIs were more likely to be infected with non-*H.pylori* bacteria than the *H.pylori*-negative patients. These findings are in line with those from previous studies showing a greater elevation of the intragastric pH in *H.pylori*-infected vs non-infected patients treated with acid inhibition^{30,31}, which in turn may cause an enhanced colonisation of the stomach with non-*H.pylori* flora³².

The bacterial species isolated from the gastric juice were mainly of oropharyngeal origin - with *Streptococci*, *Neisseria* and *Corynebacterium* as the most common species - while only a few patients harboured faecal-type flora - especially *Escherichia coli*, *Proteus* and *Klebsiella* species. The growth of faecal-like bacteria in gastric juice during pharmacological acid inhibition describes large variations among studies, ranging from very few^{15,33} to as many as 30% or more of the treated patients¹⁶. These discrepancies may reflect differences in population samples and methodologies. As an alternative or additional

explanation, a higher susceptibility to acid of such flora - compared to the oropharyngeal-like bacterial species - may prevent its isolation³³.

(a)

(b)

Figure 5.6. Non-*H.pylori* bacterial flora in gastric juice (a) and in gastric mucosa (b) in relation with the type and duration of acid inhibition.

Gastric mucosal non-*H.pylori* bacterial growth

In the current study, gastric mucosal bacterial growth was detected in about 50% of the patients on acid inhibition, mainly after long-term treatment. Of particular note was the observation that non-*H.pylori* bacteria developed with a similar prevalence and density in the gastric mucosa of patients treated either with PPIs or with H₂RAs. This finding adds support to previously published data showing that intragastric bacterial growth may occur during acid inhibition with any type of effective antisecretory drug¹²⁻¹⁶.

Interestingly, nearly half of the patients with histological evidence of non-*H.pylori* bacteria, had a “sterile” gastric juice at the time of examination. As illustrated in **Figures 5.6a** and **5.6b**, this was particularly evident in patients treated with H₂RAs. It can be reasoned therefore that the fluctuations in the intragastric pH - which are far more commonly encountered during H₂RA therapy - dynamically influence the luminal bacterial growth. In contrast, the gastric mucosa during acid inhibition seems to behave as a constant “bacterial reservoir”.

The discrepancy between luminal and mucosal growth of non-*H.pylori* flora also suggests that although hypochlorhydria - with subsequent colonisation of gastric juice - is a prerequisite for the colonisation of the mucosa, the latter may depend upon additional factors. It can be speculated that on a background of compromised gastric mucosal barrier - secondary to the *H.pylori* infection - the resident non-*H.pylori* bacteria display pathogenic properties adhering to and even penetrating into the mucosa. As illustrated in **Figures 5.3b** and **5.3c**, the identification of non-*H.pylori* bacterial species on similar mucosal positions as the *H.pylori* supports this hypothesis.

Unfortunately, in the present study we did not assess the bacterial composition in the gastric mucosa by means of microbiological culture. However, Dolby *et al*²⁴ have found a similar spectrum of microorganisms in cultures of gastric juice and of gastric tissue. In view of this finding and of our own observations²⁶, it is conceivable that the similar composition of the two intragastric compartments reflects a two-way interaction: not only can the gastric juice non-*H.pylori* bacteria colonise the gastric mucosa, but with the constant renewal of the mucosa, the bacteria might flow back into the gastric lumen, as well.

General considerations and conclusions

The results of our study may trigger the question: *Is the mucosal non-*H.pylori* bacterial growth of pathogenetic significance?* It has been largely debated whether bacterial colonisation of the stomach - during several conditions associated with hypochlorhydria - increases the risk of gastric cancer through nitrosamine synthesis^{34,35}. Also, it has been

suggested that non-*H.pylori* bacteria and their by-products may act as a persistent antigenic stimulus, and thereby augment the inflammatory response induced by the *H.pylori* infection^{22,23}. The resulting cumulative damage to the mucosa caused by a *double infection of the stomach with H.pylori and non-H.pylori bacteria* may in the long term lead to development of atrophic gastritis and ultimately of gastric cancer.

The results of this study may also have relevance for clinical bacteriological research. We have shown that the sole assessment of luminal bacterial growth by culturing gastric juice - as performed in earlier studies - seems to underestimate the changes in the intragastric milieu. In addition, the semi-quantitative histological evaluation of the non-*H.pylori* flora used in this study provides information regarding the morphology and location of the bacteria, thus being a useful complement to the standard microbiological methods.

In summary, our study clearly demonstrates that the non-*H.pylori* bacterial flora not only contaminates the gastric juice but also colonises the gastric mucosa of a large proportion of patients on continuous acid inhibitory medication. This seems to be largely influenced by the infection with *H.pylori* and the length of antisecretory therapy, either with H₂RAs or with PPIs. Further studies are needed to elucidate the role of the double infection of the stomach with *H.pylori* and non-*H.pylori* bacteria in the pathogenesis of atrophic gastritis and of gastric cancer.

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Chapter 6

Double gastric infection with
Helicobacter pylori and
non-*Helicobacter pylori* bacteria
during acid-suppressive therapy:
increase of pro-inflammatory cytokines
and development of atrophic gastritis

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Abstract

Background

Long-term acid suppression accelerates the development of atrophic gastritis in *H.pylori*-positive subjects. The pathogenetic mechanism remains unclear. This study tested the hypothesis that the gastric *double infection* with *H.pylori* and non-*H.pylori* bacterial species - during acid suppression - may result in an enhanced inflammatory response, contributing to development of atrophic gastritis.

Patients & Methods

A consecutive series of patients with gastro-oesophageal reflux disease undergoing treatment with proton pump inhibitors (n=113) or histamine₂-receptor antagonists (n=37), and 76 non-treated dyspeptic controls was investigated. Gastric mucosal *H.pylori* and non-*H.pylori* bacteria, histological gastritis, *H.pylori* serology, and circulating interleukin (IL)-1 β , IL-6, and IL-8 were examined.

Results

Patients on acid suppression with either PPIs or H₂RAs had similar prevalence of *H.pylori* infection to the controls, but a higher prevalence of non-*H.pylori* bacteria (61.1% and 59.5% vs 28.9%, $P<0.0001$ and $P<0.002$ respectively). Both the presence of *H.pylori* and non-*H.pylori* bacteria were independent risk factors of atrophic gastritis (antrum: RRs, 10.1 and 5.07; corpus: RRs, 11.74 and 6.38). Simultaneous presence of *H.pylori* and non-*H.pylori* bacteria was associated with a markedly increased risk of atrophic gastritis (antrum: RR, 20.25; corpus: RR, 20.38), compatible with a synergistic effect. Furthermore, the simultaneous presence of both types of bacteria was associated with higher cytokine levels than in patients without any type of bacteria. This increase was also greater than in patients with *H.pylori* infection alone ($P<0.001$, for both IL-1 β and IL-8).

Summary & conclusions

H.pylori-positive patients on long-term acid inhibition displayed three features: non-*H.pylori* bacterial growth, increased cytokine levels, and a high risk of atrophic gastritis. We suggest that the *double infection* with *H.pylori* and non-*H.pylori* bacteria is a major factor in the development of atrophic gastritis during gastric acid inhibition.

Introduction

Identification of *Helicobacter pylori* (*H.pylori*) has considerably improved our understanding of the pathogenesis of chronic atrophic gastritis^{1,2}. Infection with *H.pylori* causes chronic active inflammation of the gastric mucosa, which may progress towards atrophic gastritis and intestinal metaplasia, conditions that increase the risk of gastric cancer^{3,4}. The clinical outcome of the infection is driven by a complex interplay between bacterial pathogenic factors, host response, and environmental factors^{5,6}.

Current evidence indicates that the anatomical distribution and severity of *H.pylori* gastritis are strongly influenced by the individual gastric acid secretory status^{7,8}. Subjects with a normal or high level of acid output are prone to develop antral predominant gastritis and duodenal ulcer disease. In contrast, subjects with a low level of acid output are prone to develop a corpus (body) predominant gastritis, leading to glandular atrophy and risk of gastric cancer. Such corpus predominant gastritis is frequently seen when *H.pylori*-infected subjects are treated with acid-suppressive medication⁹⁻¹². At present, the mechanism underlying the accelerated development of atrophic gastritis in *H.pylori*-infected patients treated with acid inhibition is still unclear. It has been suggested that an increase in antral pH impairs local growth conditions for *H.pylori*, causing a 'shift' of the bacterium to the corpus mucosa, where the acid secretion is still preserved⁹. Furthermore, decreased gastric acid secretion may impair the dilution and washout of potentially cytotoxic and pro-inflammatory agents induced by *H.pylori*, which in turn may cause mucosal damage^{11,13}.

On the other hand, acid suppression alters the intragastric milieu, favouring the colonisation by non-*H.pylori* bacterial species: aerobic and to a minor degree anaerobic, gram-positive and gram-negative bacterial strains have been consistently isolated¹⁴⁻¹⁸. The intragastric bacterial overgrowth and its potential consequences have received considerable attention in the past¹⁴. So far, it is not known whether such bacteria could play a role in the development of atrophic gastritis during acid-suppressive therapy.

Infection with *H.pylori* induces mucosal over-expression of various pro-inflammatory cytokines, such as interleukin (IL)-1 β , IL-6, IL-8, and tumour necrosis factor α ; this is accompanied by gastric morphological changes¹⁹⁻²¹. Experimental evidence shows that commensal bacteria and their by-products can also elicit cytokine production^{22,23}. In this context, the following question emerged: *Does the gastric double infection with H.pylori and non-H.pylori bacteria cause an enhanced*

inflammatory response, leading to increased risk for development of atrophic gastritis?

In a large series of patients on continuous acid-suppressive therapy and of non-treated controls, we tested this hypothesis by studying intragastric mucosal bacterial flora in relation to the profile of circulating pro-inflammatory cytokines - IL-1 β , IL-6, and IL-8 - and to gastric mucosal histology. Special attention was paid to the development of *corpus atrophic gastritis* in this clinical setting.

Patients and methods

Study population

Between January 1997 and June 1998, 226 consecutive dyspeptic patients referred for upper gastrointestinal endoscopy to an open-access unit (University Hospital Maastricht, The Netherlands) were investigated in a cross-sectional design. Case subjects were patients on continuous acid-suppressive therapy for gastro-oesophageal reflux disease (GORD), with/without Barrett oesophagus. Continuous acid-suppressive therapy was defined as at least one daily dose of either a proton pump inhibitor (PPI) or a histamine₂-receptor antagonist (H₂RA), taken either six weeks to one year (medium-term) or longer than one year (long-term), respectively. Control subjects were dyspeptic patients with a similar distribution of age and gender, who did not receive any acid-suppressive medication or antacids before referral, and who had normal endoscopic findings. Exclusion criteria for all subjects were: (1) present or past history of peptic ulcer disease; (2) previous gastric surgery and/or vagotomy; (3) prior *H.pylori* eradication therapy; (4) treatment with antimicrobial agents or prokinetic drugs within 30 days before endoscopy; (5) chronic use of NSAIDs; (6) any condition suspected or confirmed to influence the cytokine production, such as an acute infection in the previous 30 days, non-infectious inflammatory diseases, neoplasms, liver or renal diseases, use of immunosuppressive therapy. The study was approved by the Ethical Review Board of the University Hospital Maastricht and each subject gave informed consent before being enrolled into the study.

Design of the study

General clinical records and a structured interview concerning history of reflux symptoms, type, daily dose, and length of antisecretory therapy, any comorbidity / comedication, and family history of gastric cancer were obtained from all patients. Endoscopies were performed after an overnight fast, using a carefully disinfected Pentax EG-2901 instrument.

During endoscopy, fasting gastric juice pH was measured using pH paper strips with grading steps of 0.5 from pH 0 to pH 14 (Schleicher & Schüll GmbH; Dassel, Germany). Two antral biopsies (2 cm proximal to the pylorus) and four corpus biopsies (10 cm below the gastro-oesophageal junction, along the greater curvature) were sampled for histological examination. Fasting blood samples were obtained for the determination of *H.pylori* status and circulating cytokines. All blood samples were drawn between 9:00 am and 11:00 am.

Serological tests

The *H.pylori* status of the patients was determined using an immunoblot method (Helicoblot 2.0, Imphos BV, Zambon Group, Amersfoort, The Netherlands). Strips were incubated with serum samples, diluted 1:100. After washing, they were treated with anti-human IgG antibody conjugated with alkaline phosphatase and the substrate BCIP/NBT. Bands were present for 19.5, 26.5, 30, 35, 89, and 116 kD (*CagA*) proteins. *H.pylori* positivity was assessed according to the manufacturer's instructions. *H.pylori* positive and negative control sera were included each time the test was performed.

Circulating IL-1 β , IL-6, and IL-8 levels were determined by ELISA (Cytoscreen™ human IL-1 β ELISA kit, BioSource International, California, USA; IL-6 ELISA kit IBL-Hamburg, Germany, and Genzyme IL-8 ELISA kit, Cambridge, USA, respectively). The detection limit for all these assays is 1 pg/ml. The intra-assay variations of IL-1 β , IL-6, and IL-8 are less than 5%, 4.5%, and 6.5%, whilst the inter-assay variations of these cytokines are less than 7.5%, 5.5%, and 10.5%. All samples were examined in duplicate and without knowledge of the patients' clinical and histologic data.

Histology

Serial sections (4 μ m) from formalin-fixed and paraffin-embedded specimens were prepared for haematoxylin-eosin stain (H&E), modified Giemsa stain (MG), and an immunohistochemical stain (IMM).

For the detection and histological classification of the *current* intragastric bacterial flora, the IMM and MG were used in combination, as previously detailed²⁴⁻²⁶. IMM with a polyclonal antiserum (DAKO B471, ITK diagnostics BV, Uithoorn, The Netherlands) in a dilution of 1:100 was applied to detect *H.pylori*²⁴. The presence of non-*H.pylori* bacteria was screened in the MG stain - on the basis of morphology and location - and ascertained by a negative IMM stain of an adjacent section, on the same position, as detailed elsewhere^{25,26}. At least two adjacent sections of all biopsies were examined completely at 400x magnification. If necessary a 1000x magnification was used. This

technique was validated against culture methods, in groups of patients with different gastric conditions, as well as in the context of acid inhibitory treatment^{24,26}.

The histomorphologic status of the gastric mucosa was evaluated according to the Sydney classification²⁷⁻³⁰. The presence of active inflammation (*i.e.*, neutrophilic cell infiltration), chronic inflammation (*i.e.*, mononuclear cell infiltration), glandular atrophy (*i.e.*, loss of appropriate glands), and intestinal metaplasia was graded as absent, mild, moderate or severe changes. The density of non-*H.pylori* bacterial colonisation was scored semiquantitatively, as follows: grade 0 (no bacteria detected); grade 1 (occasionally bacteria detected); grade 2 (scattered bacteria identified in several high power fields); and grade 3 (large amounts detected in many high power fields)²⁸.

For the purpose of this study, the following subtypes of chronic gastritis were considered: *diffuse non-atrophic gastritis*, defined as active and/or chronic inflammation of either antral, corpus or both antral and corpus mucosa, without any signs of glandular atrophy; *antral predominant atrophic gastritis* (APAG), defined as glandular atrophy detected only in the antrum or in both antrum and corpus, but with a higher degree of active or chronic inflammation in the antrum; and *corpus predominant atrophic gastritis* (CPAG), defined as glandular atrophy detected only in the corpus or in both antrum and corpus, but with a higher degree of active or chronic inflammation in the corpus. All biopsy specimens were reviewed by one experienced gastroenterological pathologist (A.B.) and by the main investigator (S.S.), without knowledge of the patients' clinical and serological data. In the event of discordant results (less than 5% of all cases), the specimens were re-examined by a second pathologist and were discussed until agreement was reached.

Statistical evaluation

Differences in dichotomous variables were examined using the χ^2 test or Fisher's exact test, when appropriate. Differences in continuous variables were examined using the Kruskal-Wallis test for multiple comparisons and the Mann-Whitney *U* test for two group comparisons. Correlations were performed with the Spearman rank test. The concordance between histological (IMM) and serological evidence of *H.pylori* was tested with *k* statistics (*k* index). Univariate analyses using as outcome variable the presence of atrophic gastritis were computed for each potential risk factor: age, gender, duration of acid-suppressive therapy, *CagA* positivity, presence of *H.pylori* alone, of non-*H.pylori* alone, and of both types of bacteria. Multiple logistic regression analyses were carried out to determine the independent contribution of each factor to the presence of atrophic gastritis³¹. Interaction was

studied with standard methods³². The *relative excess risk* due to an interaction was calculated as: relative risk (A and B) - relative risk (A without B) - relative risk (B without A) + 1, where A and B are the factors whose interaction is being studied. The *synergy index* was calculated as: [relative risk (A and B) - 1] / [relative risk (A without B) + relative risk (B without A) - 2]. In the multiple regression analysis, a relative excess risk > 0 and a synergy factor > 1 were regarded as indices of interaction between two risk factors. Two-sided P-values < 0.05 were assumed to indicate statistical significance.

Results

Table 6.1 shows the demographic, clinical, and endoscopic characteristics of the 226 patients, classified according to the type of acid-suppressive therapy they were undergoing. The age distribution and sex ratios were comparable among the three patient groups: non-treated controls, H₂RA group, and PPI group.

Median gastric juice pH levels in *H.pylori*-positive patients were 3.1 (2.5-3.8) in non-treated controls, 4.0 (2.9-4.9) in the H₂RA group, and 5.6 (4.8-6.5) in the PPI group; the corresponding median gastric juice pH levels in *H.pylori*-negative patients were 2.2 (1.8-2.7), 3.0 (2.3-3.6), and 4.8 (4.2-5.2), respectively.

Table 6.1. Demographic, clinical and endoscopic characteristics of the 226 patients enrolled into the study.

Characteristics	Controls	Acid inhibition	
		H ₂ RA group	PPI group
Number of patients	76	37	113
Age (years)*	51.5(19-79)	52(20-73)	51(21-78)
Male:female ratio	1.05:1	0.95:1	1.3:1
Duration of acid inhibition (months)*	-	32(1.5-216)	29(1.5-168)
Dose of medication (mg daily)*	-	300(150-450)†	40(20-80)‡
First degree relatives with gastric cancer (%)	2.6	2.7	3.5
Endoscopic findings (%)			
- Barrett oesophagus	-	5.5	29.2
- erosive oesophagitis	-	32.4	15.0
- normal oesophagus	100	62.1	55.8

*Data are expressed as median (range). Daily doses of ranitidine† and of omeprazole‡.

Prevalence and distribution of intragastric bacteria in the gastric mucosa

Figure 6.1 illustrates the prevalence of *H.pylori* and non-*H.pylori* bacteria in the gastric mucosa. The patients on acid suppression with either PPIs or H₂RAs had similar prevalence of *H.pylori* infection to non-treated controls, but a higher prevalence of non-*H.pylori* bacteria (61.1% and 59.5% vs 28.9%, $P<0.0001$ and $P<0.002$). No significant difference in this regard was found between the PPI group and the H₂RA group.

Figure 6.1. Prevalence of *H.pylori* and non-*H.pylori* bacteria in the gastric mucosa of 226 subjects enrolled into the study.

The distribution of *H.pylori* between antral and corpus mucosa was similar in patients treated with H₂RAs and controls, whilst patients treated with PPIs had a lower prevalence of *H.pylori* colonisation in antral than corpus mucosa (27.4% vs 34.5%, $P=0.033$) (**Table 6.2**). There were no significant differences in prevalence of non-*H.pylori* bacteria between antral and corpus mucosa in each of the three patient groups.

The patients on acid suppression with either PPIs or with H₂RAs had also higher median scores (grade 1-3) of non-*H.pylori* bacterial density - in both the antrum and corpus mucosa - than the controls [antrum:

P=0.012 and P=0.003; corpus: P=0.05 and P=0.028, respectively] (Table 6.2). No significant differences in this regard were found between the PPI group and the H₂RA group, or between antral and corpus mucosa within each of the three patient groups.

Table 6.2. Distribution pattern and density of colonisation by *H.pylori* and non-*H.pylori* bacteria.

	Controls	Acid inhibition	
		H ₂ RA group	PPI group
<i>H.pylori</i>			
antral mucosa			
prevalence (%)	40.8	37.8	27.4*
density (median score)	1 (1-3)	1 (1-3)	1 (1-3)
corpus mucosa			
prevalence (%)	39.5	37.8	34.5
density (median score)	1 (1-3)	1 (1-3)	1 (1-3)
Non-<i>H.pylori</i> bacteria			
antral mucosa			
prevalence (%)	25.0	48.6	46.9
density (median score)	1 (1-3)	2 (1-3)†	2 (1-3)†
corpus mucosa			
prevalence (%)	23.7	56.8	52.2
density (median score)	1 (1-3)	2 (1-3)†	2 (1-3)†

* Lower prevalence of *H.pylori* in the antral vs corpus mucosa in patients treated with PPIs (P=0.033).

† Higher density of non-*H.pylori* bacteria in patients treated with PPIs or with H₂RAs than in the controls (antrum: P=0.012 and P=0.003; corpus: P=0.05 and P=0.028).

Intragastric bacteria in relation to chronic gastritis

Overall, 39 (17.2%) patients had diffuse non-atrophic gastritis, 44 (19.5%) patients had antral predominant atrophic gastritis (APAG), and 28 (12.4%) had corpus predominant atrophic gastritis (CPAG), whilst the remaining 115 (50.9%) patients had normal gastric histology. In patients with APAG, antral gland atrophy was mild in 22 cases, moderate in 14 cases, and severe in 8 cases. In patients with CPAG, corpus gland atrophy was mild in 6 cases, moderate in 12 cases, and severe in 10 cases. Of the 44 patients with APAG, 38 had intestinal metaplasia, which in 28 cases was mild, in 6 cases was moderate, and in the remaining 4 was severe. Of the 28 patients with CPAG, 20 had intestinal metaplasia, which in 10 cases was mild, in 6 cases was moderate, and in 4 cases was severe.

In patients with APAG, *H.pylori* only, non-*H.pylori* only and both types of bacteria were detected in 15 (34.1%), 10 (22.7%), and 17 (38.6%) of the 44 cases, whereas the corresponding figures in patients with CPAG were 9 (32.1%), 7 (25%), and 11 (39.3%) of the 28 cases, respectively.

Overall, there was strong concordance between histology (IMM) and serology for the presence of *H.pylori* (controls, 95% agreement, $k=0.89$; H₂RAs, 94% agreement, $k=0.84$; PPIs, 90% agreement, $k=0.81$). *CagA* antibodies were found in 62 (69.7%) of the histologically *H.pylori*-positive subjects (22 of 32 [68.8%] controls, 11 of 15 [73.3%] patients on H₂RAs, and 29 of 42 [69%] patients on PPIs). In histologically *H.pylori*-positive subjects, APAG was more common in *CagA*-positive than *CagA*-negative subjects ($P=0.024$), but no such relation was found between *CagA* positivity and CPAG ($P=0.42$).

In **Table 6.3**, the results of the multiple logistic regression analysis examining independent risk factors for the development of atrophic gastritis are detailed. Age was associated with a minor - but significant - risk of atrophic gastritis, whilst gender did not influence this outcome. In contrast to antral atrophic gastritis, the risk of corpus atrophic gastritis increased with the duration of acid inhibition, as expressed per year of treatment. The corresponding relative risk after ten years of therapy can be estimated at 3.16 (95% CI, 2.79-3.58). *CagA* positivity was a risk factor for antral atrophic gastritis, but not for corpus atrophic gastritis.

Table 6.3. Results of multivariate analyses to determine the independent risk factors of atrophic gastritis in antrum and corpus.

Risk factor	Adjusted odds ratio (95% CI)		P-value
Outcome: antral predominant atrophic gastritis			
Age (per decade)	1.04	(1.01-1.07)	0.018
Gender (female vs male)	0.86	(0.42-1.80)	0.70
Duration of acid-suppressive therapy (years)	1.07	(0.95-1.22)	0.24
CagA positivity	2.67	(1.12-6.41)	0.028
H.pylori vs no bacteria	10.10	(2.14-47.61)	0.003
Non-H.pylori vs no bacteria	5.07	(1.05-24.40)	0.043
Both H.pylori and non-H.pylori vs no bacteria*	20.25	(4.27-96.08)	0.0002
Outcome: corpus predominant atrophic gastritis			
Age (per decade)	1.04	(1.01-1.07)	0.024
Gender (female vs male)	0.51	(0.21-1.26)	0.40
Duration of acid-suppressive therapy (years)	1.12	(1.02-1.30)	0.021
CagA positivity	1.6	(0.4-5.8)	0.62
H.pylori vs no bacteria	11.74	(1.41-97.80)	0.022
Non-H.pylori vs no bacteria	6.38	(0.78-53.89)	0.088
Both H.pylori and non-H.pylori vs no bacteria†	20.38	(2.46-168.55)	0.005

* Relative excess risk of APAG due to the interaction between *H.pylori* and non-*H.pylori* bacteria=6.08; synergy index=1.46.

† Relative excess risk of CPAG due to the interaction between *H.pylori* and non-*H.pylori* bacteria=3.26; synergy index=1.26.

H.pylori infection alone was an independent risk factor of atrophic gastritis in both antrum and corpus. The presence of non-*H.pylori* bacteria alone independently increased the risk of antral atrophic

gastritis, but only as a trend in the gastric corpus. The simultaneous presence of *H.pylori* and non-*H.pylori* bacteria was associated with the highest risk of atrophic gastritis in antrum and corpus when compared to the risk by either type of bacteria alone. The *relative excess risk* due to the interaction between *H.pylori* and non-*H.pylori* bacteria was 6.08 for APAG and 3.26 for CPAG, compatible with a synergistic effect (*synergy index* of 1.46 and 1.26, respectively). Analysis of the data based on serological - instead of histological - evidence of *H.pylori* provided similar results (data not shown).

Circulating cytokines in relation to intragastric bacteria and chronic gastritis

In the entire series, four subgroups of patients were distinguished according to the presence of *H.pylori* and of non-*H.pylori* bacteria in the gastric mucosa: patients without any type of bacteria (64 cases), with *H.pylori* alone (49 cases), with non-*H.pylori* bacteria alone (73 cases), and with both types of bacteria (40 cases). Patients infected with *H.pylori* alone had higher levels of IL-1 β and IL-8 (**Figures 6.2A and 6.2B**) than those without any type of bacteria ($P < 0.0001$, for both cytokines). Patients with non-*H.pylori* bacteria alone had slightly higher IL-1 β and IL-8 levels than those without any type of bacteria, but differences reached statistical significance for IL-8 only ($P < 0.05$). Patients harbouring both *H.pylori* and non-*H.pylori* bacteria had threefold higher levels of circulating IL-1 β and IL-8 than those without any type of bacteria ($P < 0.0001$, for both cytokines); this increase was also greater than that in patients with *H.pylori* infection alone ($P < 0.001$, for both cytokines).

Table 6.4 describes the relationship between cytokine levels and different types of chronic gastritis. Patients with diffuse non-atrophic gastritis, patients with APAG, and especially those with CPAG had significantly higher circulating IL-1 β , IL-6, and IL-8 levels than those with normal gastric histology. As illustrated in **Figure 6.3**, in patients with APAG or with CPAG, cytokine levels - particularly IL-8 - were highest in patients with *double infection* and *severe* atrophic gastritis. As shown in **Figure 6.4**, in patients with CPAG, cytokine levels positively correlated with the histological degree of glandular atrophy. Also, *CagA*-positive subjects had significantly higher IL-8 levels than *CagA*-negative subjects (3.6 [0-12.9] pg/ml vs 2.4 [0-10.4] pg/ml, $P = 0.012$), but no such relation was found between *CagA* positivity and IL-1 β ($P = 0.12$) or IL-6 ($P = 0.26$) levels.

Figure 6.2. Individual values of circulating IL-1 β (a) and IL-8 (b) in subjects with *H.pylori* alone, non-*H.pylori* alone, or with both types of bacteria compared to those without bacteria in the gastric mucosa. Median values are shown by horizontal lines. Numbers of subjects in parentheses.

Table 6.4. Circulating cytokines in relation to different types of chronic gastritis.

	Circulating IL-1 β (pg/ml)	Circulating IL-6 (pg/ml)	Circulating IL-8 (pg/ml)
Normal gastric histology (n=115)	1.5 (0-4.2)	0.8 (0-7)	1.7 (0-5.5)
Diffuse non-atrophic gastritis (n=39)	2.3 (0.3-7.3)**	1.4 (0.1-7)**	3.2 (0-12.9)**
Antral predominant atrophic gastritis (APAG, n=44)	2.3 (0.1-5.5)*	1.1 (0-3.8)	2.6 (0-10.8)*
Corpus predominant atrophic gastritis (CPAG, n=28)	3.3 (0.1-6.5)***	1.9 (0.6-17.3)***	5.6 (0.1-12.9)***

Patients with diffuse non-atrophic gastritis, patients with APAG, but especially those with CPAG had higher circulating cytokine levels than those with normal gastric histology. Values are expressed as medians (interquartile ranges).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs those with normal gastric histology.

Figure 6.3. Relationship between intragastric bacteria, circulating cytokines (IL-8 level), and the histological degree of atrophic gastritis. Median IL-8 values are shown by *columns*. Numbers of subjects in parentheses. Patients with APAG and with CPAG are shown together.

Figure 6.4. Correlation between circulating IL-1 β ($\square$), IL-6 ($\circ$), IL-8 ($\triangle$), and the histological degree of corpus predominant atrophic gastritis. Numbers of subjects in parentheses.

Figure 6.5 shows the relationship between presence of non-*H.pylori* bacteria and of corpus predominant atrophic gastritis (*left scale*), cytokine levels (*right scale*), and duration of treatment with PPIs, in *H.pylori*-negative (**A**) and -positive (**B**) subjects on acid inhibition vs *H.pylori*-negative and -positive non-treated controls. Five subgroups of patients were distinguished according to the length of treatment they had undergone (from 'none' to '>36 months'). Age distribution was similar across the five subgroups of patients with or without *H.pylori* infection. In *H.pylori*-negative patients treated with PPIs, the prevalence of non-*H.pylori* bacteria increased with the longer duration of treatment; this was paralleled by a low prevalence of corpus atrophic gastritis (affecting 1 out of 44 [2.2%] controls, and 1 out of 71 [1.4%] patients on PPIs), and by nearly stable cytokine levels. In contrast, in *H.pylori*-positive patients treated with PPIs, the prevalence of non-*H.pylori* bacteria increased with the longer duration of treatment, and was paralleled by increasing prevalence of corpus atrophic gastritis (affecting 9 out of 10 [90%] patients on PPIs longer than 36 months), and by increasing cytokine levels. Similar figures applied to both types of acid-suppressive therapy (PPIs and H₂RAs), but these reached statistical significance for the PPI group only, probably because of the small number of patients in the H₂RA group (figure not shown).

Higher prevalence of non-*H. pylori* bacteria (* $P < 0.01$) vs controls and vs patients treated <12 months.
No significant differences in the prevalence of CPAG or the cytokine levels.

Higher prevalence of non-*H. pylori* bacteria (* $P < 0.01$), higher prevalence of CPAG († $P < 0.01$),
and higher cytokine levels ($P < 0.05$ for all cytokines) vs controls and vs patients treated <12 months.

Figure 6.5. Relationships between the presence of non-*H. pylori* bacteria (■), of corpus predominant atrophic gastritis (■) (left scale), cytokine levels (right scale), and duration of treatment with PPIs in *H. pylori*-negative (A) and -positive (B) subjects on acid inhibition vs *H. pylori*-negative and -positive non-treated controls. Median levels of circulating IL-1β (□), IL-6 (○), and IL-8 (△) are represented.

Discussion

This study addressed the question whether the *double gastric infection* with *H.pylori* and non-*H.pylori* bacteria - in patients treated with acid-suppressive medication - may result in an enhanced inflammatory response, contributing to an increased risk of developing atrophic gastritis. We have shown that: i) *H.pylori*-infected patients on long-term acid inhibition displayed three features: non-*H.pylori* bacterial growth, increased cytokine levels, and a high prevalence of corpus atrophic gastritis; ii) the development of atrophic gastritis during acid-suppressive therapy was associated with a simultaneous gastric infection with *H.pylori* and non-*H.pylori* bacteria; iii) the type of interaction between *H.pylori* and non-*H.pylori* bacteria was *synergistic*. Taken together, these findings indicate that the double gastric infection with *H.pylori* and non-*H.pylori* bacteria may play a major role in the development of atrophic gastritis.

Intragastric bacteria, atrophic gastritis, and circulating cytokines

Intragastric growth of non-*H.pylori* bacteria commonly occurs at elevated intragastric pH, during administration of either H₂RAs or PPIs¹⁵⁻¹⁸. Our results confirm and further extend this observation, demonstrating that, in *H.pylori*-positive subjects treated with acid inhibition, the overgrowth of non-*H.pylori* bacteria was accompanied by a high rate of corpus atrophic gastritis. This latter phenomenon, however, was not observed in the *H.pylori*-negative patients treated in a similar fashion. As such, it can be argued that infection with *H.pylori* is a *prerequisite* for the development of atrophic gastritis in this setting^{10,11}.

An important finding of this study was that cytokine levels correlated with the anatomical distribution and the severity of chronic gastritis. Patients with antral predominant atrophic gastritis and those with diffuse non-atrophic gastritis displayed a slight - but significant - elevation in circulating cytokines compared to patients with normal mucosa. Noteworthy, the predominant involvement of the gastric body and fundus - representing a (twofold) larger anatomical area than the gastric antrum - was associated with the greatest elevation in circulating cytokines.

Our results also indicated that increasing age was associated with a higher risk of atrophic gastritis. Yet, its impact was marginal compared to that of the *H.pylori* infection, thereby supporting the contention that the development of atrophic gastritis is merely *H.pylori*-dependent than age-dependent^{33,34}. Additionally, we found that infection with *CagA*-positive *H.pylori* strains increases the risk of atrophic gastritis in the antrum but not in the corpus, a finding in line with recently published data³⁵.

Non-*H.pylori* bacteria: a cause or a consequence of atrophic gastritis?

Two possible explanations can be suggested for the demonstrated link between non-*H.pylori* bacteria and atrophic gastritis:

1. Non-*H.pylori* bacterial overgrowth could be an *epiphenomenon*. Previous studies, including ours, have shown that in subjects infected with *H.pylori*, acid-suppressive therapy leads to a greater elevation in gastric juice pH^{8,36,37}, predisposing to enhanced colonisation of the stomach by non-*H.pylori* bacterial species^{37,38}. This observation has been confirmed in the present study. Furthermore, recent evidence linked genetic factors - such as polymorphisms in the genes encoding IL-1 β - to an enhanced cytokine production in response to *H.pylori* infection, resulting in development of atrophic gastritis³⁹, and subsequent overgrowth of non-*H.pylori* bacteria.
2. Non-*H.pylori* bacterial flora could be a *co-factor* in the development of atrophic gastritis. The following arguments come in favour of this hypothesis: First, as shown in **Figure 6.5(B)**, *H.pylori*-positive patients on long-term PPIs were characterised by non-*H.pylori* bacterial growth and a high prevalence of corpus atrophic gastritis; this was in sharp contrast to the *H.pylori*-positive non-treated controls, who had similar demographic features, but a very low rate of corpus atrophic gastritis. Second, in *H.pylori*-positive patients treated with acid suppression, the presence of non-*H.pylori* bacteria *preceded* in time the development of corpus atrophic gastritis, a sequence supporting the hypothesis that bacterial infection was a *causal factor* rather than a *consequence* of atrophy. Third, the magnitude and ranking of cytokine increase were strongly concordant with the risk factors for atrophic gastritis emerging from the multiple regression analysis: patients harbouring the *double infection* had the highest cytokine levels paralleled by the highest risk of atrophic gastritis.

Atrophic gastritis during acid-suppressive therapy: possible mechanism(s)

It remains to be explained how the gastric co-infection with non-*H.pylori* bacteria could cause aggravation of the *H.pylori*-induced chronic gastritis. A study by Mowat *et al*³⁸ has indicated that acid inhibition with omeprazole profoundly alters the intragastric milieu in subjects infected with *H.pylori*, leading to enhanced bacterial colonisation, elevated intragastric nitrites, and depletion of intragastric vitamin C. They

suggested that such changes may facilitate the development of gastric mucosal atrophy. Additionally, it is possible that commensal bacteria and their by-products act as a persistent antigenic stimulus, that enhances and perpetuates the inflammatory response initiated by the *H.pylori* infection. In this sense, it is worthwhile to notice that the lipopolysaccharides (LPS) produced by gram-negative aerobic bacteria, such as *Escherichia coli*, may evoke substantially (>1000-fold) greater pro-inflammatory response than the *H.pylori* LPS^{40,41}. The pro-inflammatory cytokines - mainly IL-1 β - are potent inhibitors of the gastric parietal cell secretion⁴²⁻⁴⁴. As such, the increased production of IL-1 β - driven by a *double infection* - may further promote the overgrowth of non-*H.pylori* bacteria, in a self-perpetuating process. Such explanations could underlie the observed synergistic interaction between *H.pylori* and non-*H.pylori* bacteria.

Figure 6.6 suggests a possible sequence of events leading to the accelerated development of atrophic gastritis during acid-suppressive therapy: In the presence of *H.pylori* infection, long-term acid-suppressive therapy leads to a corpus predominant pattern of gastritis or pangastritis^{9-11,45}. Chronic hypochlorhydria and subsequent superinfection with non-*H.pylori* species may occur. This double gastric infection with *H.pylori* and non-*H.pylori* bacteria may then elicit a greater inflammatory response - as reflected by the elevated cytokines - leading to an accelerated development of atrophic gastritis.

Figure 6.6. Development of atrophic gastritis during acid-suppressive therapy: a model.

Methodological aspects

A few comments have to be made concerning methodological aspects of this study: First, the patients were consecutively recruited from a primary-care population, homogenous with regard to risk factors for the development of atrophic gastritis. Hence, the results presented and conclusions are representative for an everyday clinical situation. Second, this study was cross-sectional in design. Nevertheless, the role of the time factor in the development of atrophic gastritis could be estimated by patient histories and documentation concerning the length of antisecretory treatment, and by comparison with a sizeable control group. Further longitudinal studies are needed, however, to fully elucidate the time-course of gastric morphological changes caused by a double bacterial infection. Third, in the present study no special attention was paid to further identify the non-*H.pylori* bacterial species in the gastric mucosa, as this has been done in earlier publications by our own group^{24,26}; we have consistently found a predominance of the oropharyngeal flora (e.g., *Streptococci* spp., *Neisseria* spp., and *Corynebacterium* spp.), but faecal-type bacteria (e.g., *Escherichia coli*, *Klebsiella* spp.) have been also isolated.

General considerations and summary

The present study may have clinical implications for the treatment of *H.pylori*-positive patients subjected to long-term acid-suppressive medication for acid-related disorders, especially for gastro-oesophageal reflux disease. *H.pylori* 'test-and-treat' strategy seems recommendable in this setting, in order to prevent the potential cumulative damage to the gastric mucosa due to a double bacterial infection. The non-*H.pylori* infection alone - unavoidable during gastric acid suppression - seems to do less harm to the gastric mucosa, and probably does not require major concern.

In summary, we have shown that in *H.pylori*-positive patients, the gastric *co-infection* with non-*H.pylori* bacteria - secondary to acid inhibition - seems to elicit a more vigorous inflammatory response. This may evolve to corpus atrophic gastritis, which is a precursor lesion for gastric cancer. The demonstrated relation between gastric acid inhibition, *H.pylori*, and non-*H.pylori* bacteria suggests that closer attention should be paid to the interaction between these factors when investigating the mechanisms underlying the development of atrophic gastritis and gastric cancer.

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Chapter 7

Prediction of atrophic body gastritis by serum markers

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Abstract

Background & Aims

Elevated serum gastrin and a low pepsinogen A:C ratio are well-recognized markers of atrophic body gastritis. We have shown that the presence of body atrophy is also associated with elevated serum pro-inflammatory cytokines. This study tested the hypothesis that serum cytokines may have additional value to gastrin and pepsinogens in screening for atrophic body gastritis.

Methods

Two hundred and twenty-six consecutive patients were investigated on referral for upper gastrointestinal endoscopy: 150 were patients with gastro-oesophageal reflux disease, receiving acid inhibitory medication either with proton pump inhibitors (n=113) or with histamine₂-receptor antagonists (n=37), and 76 were non-treated controls, who had normal endoscopic findings. Gastric mucosal biopsies were examined for histological evaluation of gastritis (Sydney classification). Serum samples were analysed for gastrin, chromogranin A (CgA), and pepsinogens A and C by RIA, and for the interleukins (IL)-1 β , IL-6, and IL-8 by ELISA.

Results

Subjects with atrophic body gastritis had significantly higher serum gastrin ($P<0.01$) and serum CgA ($P<0.01$) levels, and significantly lower pepsinogen A:C ratios ($P<0.001$) than those without atrophic body gastritis. Additionally, serum IL-1 β , IL-6, and IL-8 levels were significantly higher in subjects with than in those without atrophic body gastritis ($P<0.0001$, for all cytokines). To optimise the detection of body atrophy we defined the atrophic body gastritis (**ABG index**) - that is the ratio between the simultaneously measured IL-8 and pepsinogen A:C levels. The area under the ROC curve for **ABG index** was significantly greater than that for serum gastrin and for serum pepsinogen A:C alone (0.91 ± 0.029 vs 0.72 ± 0.042 , and vs 0.83 ± 0.031 , $P=0.018$ and $P=0.049$). Using the **ABG index** at a cut-off value of 1.8 pg/ml, 91% of the cases were classified correctly.

Conclusions

The simultaneous presence of a high IL-8 level and a low pepsinogen A:C ratio accurately predicts the atrophic body gastritis. Besides indicating presence of body atrophy, the **ABG index** seems to reflect *ongoing chronic active inflammation*, which in long-term may promote the development of gastric cancer.

Introduction

Chronic atrophic gastritis is a multifactorial condition, caused by the interplay between a genetic set-up, gastric bacterial infection, and environmental factors¹⁻⁵. It is recognised that atrophic body gastritis increases the risk of developing non-cardia gastric cancer⁶⁻⁸. The identification of this lesion and its causes is therefore of considerable importance, making it possible to prevent this major cause of cancer death. Several studies addressed the potential of non-invasive, serologic screening for advanced **atrophic body gastritis (ABG)**; such cases would prompt further endoscopic and histologic investigation. Currently, serum gastrin and serum pepsinogens A and C are considered useful predictors of the morphologic and functional status of the gastric body mucosa⁹⁻¹¹: elevated serum gastrin and a low pepsinogen A:C ratio commonly develop in the presence of body mucosal atrophy¹²⁻¹⁶. Additionally, serum chromogranin A (CgA) may increase, reflecting concomitant hyperfunction and proliferation of the fundic enterochromaffin-like (ECL) cells^{17,18}.

Recent results of our group have indicated that the presence and severity of the *Helicobacter pylori* (*H.pylori*)-induced atrophic body gastritis positively correlate to serum pro-inflammatory cytokine levels¹⁹. However, no studies have been conducted, so far, to evaluate whether the assessment of serum cytokines in this context may provide additional information to the known predictors of atrophic body gastritis.

In a prospective cohort of dyspeptic subjects referred for upper gastrointestinal endoscopy to an open-access unit, we have compared the clinical utility of measuring serum pro-inflammatory cytokines: interleukin (IL)-1 β , IL-6, and IL-8, together with serum gastrin and serum pepsinogens in screening for atrophic body gastritis. As a result, we propose a simple and reliable screening test - the **atrophic body gastritis (ABG) index** - to optimise the detection of this condition.

Patients and methods

Two hundred and twenty-six consecutive dyspeptic patients referred for upper gastrointestinal endoscopy to an open access unit (121 men and 105 women; mean age, 50.9 years, range, 19-79 years) were investigated. The study population comprised 150 patients receiving acid-suppressive therapy - either histamine₂-receptor antagonists (H₂RAs, n=37) or proton pump inhibitors (PPIs, n=113) - for gastro-oesophageal reflux disease (GORD), and 76 dyspeptic subjects,

comparable for age and gender, who did not receive acid-suppressive medication before referral, and who had normal endoscopic findings. All patients previously participated in a study investigating the relationship between intragastric bacteria and the development of atrophic gastritis during acid-suppressive therapy¹⁹. The following exclusion criteria were applied in all patients: present or past history of peptic ulcer disease; previous gastric surgery and/or vagotomy; prior *H.pylori* eradication therapy; treatment with antimicrobial agents, chronic NSAIDs use; any condition suspected or confirmed to influence the cytokine production, such as an acute infection in the previous 30 days; non-infectious inflammatory diseases, neoplasms, chronic liver or renal diseases, use of immuno-suppressive therapy. The study was approved by the Ethical Review Board of the University Hospital Maastricht, The Netherlands, and each subject gave informed consent before entering into the study.

Endoscopy was performed after an overnight fast, using a Pentax EG-2901 instrument. During endoscopy two antral biopsies (2 cm proximal to the pylorus) and four corpus biopsies (10 cm below the gastro-oesophageal junction, along the greater curvature) were sampled for histological examination. Fasting blood samples were obtained for the measurement of serum gastrin, CgA, and pepsinogens, as well as of serum IL-1 β , IL-6 and IL-8. All blood samples were drawn between 9:00 am and 11:00 am.

Serum assays

Serum gastrin was measured by radioimmunoassay, using antibodies raised in rabbits against synthetic unsulphated human gastrin 2-17 covalently coupled to bovine serum albumin, as described previously²⁰. The antibodies bind to all major forms of circulating gastrin, with almost equal potency. Serum CgA was measured by radioimmunoassay, using polyclonal antibodies raised in rabbits against a fragment of chromogranin A (CgA 116-439) purified from the urine of a patient with a carcinoid tumour^{21,22}. Serum pepsinogen A and serum pepsinogen C were measured by radioimmunoassay, using antibodies raised in goats against pepsinogens purified from human gastric resection specimens, as detailed elsewhere^{23,24}. The upper limit of the normal range for serum gastrin was <85 ng/L, and for serum CgA was <4 nmol/L; the normal level for pepsinogen A ranges from 19 to 95 μ g/L; for pepsinogen C, from 2 to 24 μ g/L; and for the pepsinogen A:C ratio, from 2.1 to 14.1. The total assay variations with these methods are less than 10% for serum gastrin, less than 6.4% for serum CgA, and less than 10% for both pepsinogen A and C.

Serum IL-1 β , IL-6, and IL-8 were measured by ELISA (Cytoscreen™ human IL-1 β ELISA kit, BioSource International, California, USA; IL-6

ELISA kit IBL-Hamburg, Germany, and Genzyme IL-8 ELISA kit, Cambridge, USA). The detection limit for all these assays is 1 pg/ml. Intra-assay variations of IL-1 β , IL-6, and IL-8 were less than 5%, 4.5%, and 6.5%, whereas the inter-assay variations were less than 7.5%, 5.5%, and 10.5%, respectively. All samples from the same patient were analysed in duplicate and without knowledge of the patients' clinical and histologic data.

Histology

Serial sections (4 μ m) of formalin-fixed and paraffin embedded biopsy specimens were stained with haematoxylin-eosin for general histological analysis. The histomorphologic status of the gastric body mucosa was assessed according to the Sydney classification^{25,26}. The presence of active inflammation (*i.e.*, the amount of neutrophilic cell infiltration), chronic inflammation (*i.e.*, amount of mononuclear cell infiltration), gland atrophy (*i.e.*, loss of oxyntic glands), and intestinal metaplasia in the gastric body mucosa was graded from 0 to 3 (0, absent; 1, mild; 2, moderate; 3, severe changes). For the purpose of this study, atrophic body gastritis (**ABG**) was defined as presence of oxyntic glands loss accompanied by at least mild chronic and/or active inflammation of the body mucosa.

In addition, specimens of body mucosa were immunostained for endocrine cells, using a monoclonal antibody against human CgA²⁷. The status of the ECL cell population of the body mucosa was assessed according to the Solcia classification²⁸; endocrine hyperplastic lesions were graded from 0 to 3 (0, absent; 1, diffuse hyperplasia; 2, linear hyperplasia; and 3, micronodular hyperplasia). All biopsy specimens were reviewed by one experienced gastroenterological pathologist (A.B.) and by the main investigator (S.S.), without knowledge of the patients' clinical and serologic data. In the event of discordant results, the specimens were re-examined by a second pathologist and a joint diagnosis was made.

Helicobacter pylori

Determination of current *H.pylori* status was performed in both antral and body mucosa by modified Giemsa stain, immunohistochemistry (IMM), and culture. For the IMM, a purified polyclonal antiserum (DAKO B471, ITK diagnostics BV, Uithoorn, The Netherlands) in a dilution of 1:100 was used²⁹. Culture of gastric biopsies was performed on selective *Campylobacter* plates, under microaerophilic conditions. *H.pylori* was considered present when histopathology and/or culture were positive.

Statistical analysis

Differences in dichotomous variables were examined using the χ^2 test or Fisher's exact test when necessary. Differences in continuous variables were examined using the Kruskal-Wallis test for multiple comparisons and the Mann-Whitney U test for two group comparisons. Correlations were performed with the Spearman rank test. Receiver operating characteristics (ROC) curves and the respective areas under the curves (AUC) were computed for each potential predictor of atrophic body gastritis. The magnitude of the AUC provides information about the test accuracy. All combinations between serum cytokines, gastrin, and pepsinogens have been tested in an attempt to optimize the diagnostic accuracy for atrophic body gastritis. As such, we calculated the ratio between IL-8 and pepsinogen A:C, which was defined as the **atrophic body gastritis index (ABG index = IL-8 / pepsinogen A:C)**. Non-parametric estimates of the areas under the ROC curves and their standard errors (SE) were applied³⁰. A procedure involving paired data was used to compare areas under the ROC curves³¹. The best cut-off was considered as the value that maximized the likelihood ratio (LR) obtained using the following formula: $LR = (\text{probability of true positive} + \text{probability of true negative}) / (\text{probability of false positive} + \text{probability of false negative})$ ³². Using these cut-off values, the sensitivity, specificity, and the diagnostic accuracy (percentage of correctly predicted results) were also assessed. All statistical evaluations were performed running the SPSS 10.0, for Windows. Two-sided P-values < 0.05 were assumed to indicate statistical significance.

Results

Table 7.1 describes the histomorphologic status of the gastric body mucosa in subjects across the three subgroups: non-treated controls, H₂RA group, and PPI group. Overall, *H.pylori*-positive subjects had significantly higher prevalence of atrophic body gastritis (38 out of 89 [42.7%] vs 4 out of 137 [2.9%], $P < 0.0001$), and of ECL cell hyperplasia (21 out of 89 [23.6%] vs 6 out of 137 [4.4%], $P < 0.0001$) than the *H.pylori*-negative subjects. In the 42 subjects with atrophic body gastritis, gland atrophy was accompanied by chronic inflammation in all cases (10 mild; 22 moderate; 10 severe), and/or active inflammation in 22 cases (16 mild; 4 moderate; 2 severe).

Table 7.1. Histomorphologic status of the gastric body mucosa in *H. pylori*-negative and *H. pylori*-positive patients on acid-suppressive therapy and in non-treated controls.

Histologic parameter	Controls		H ₂ RA group		PPI group	
	<i>H. pylori</i> (-) (n=44)	<i>H. pylori</i> (+) (n=32)	<i>H. pylori</i> (-) (n=22)	<i>H. pylori</i> (+) (n=15)	<i>H. pylori</i> (-) (n=71)	<i>H. pylori</i> (+) (n=42)
Active inflammation						
Grade 1;2;3*	2 (4.6%) 1;0;1	22 (68.7%) 13;4;5	0 (0%) 0;0;0	13 (86.7%) 8;4;1	4 (5.6%) 2;2;0	35 (83.3%) 24;10;1
Chronic inflammation						
Grade 1;2;3*	2 (4.6%) 1;1;0	24 (74%) 18;4;2	1 (4.5%) 1;0;0	9 (60%) 6;2;1	4 (5.6%) 1;2;1	42 (100%) 22;12;8
Gland atrophy						
Grade 1;2;3*	1 (2.3%) 1;0;0	8 (25%) 4;2;2	1 (4.5%) 1;0;0	7 (46.7%) 3;2;2	2 (2.8%) 2;0;0	23 (54.7%) 9;8;6
Intestinal metaplasia						
Grade 1;2;3*	0 (0%) 0;0;0	2 (6.3%) 1;1;0	0 (0%) 0;0;0	4 (26.7%) 2;1;1	1 (1.4%) 1;0;0	18 (42.8%) 10;6;2
ECL cell hyperplasia						
Grade 1;2;3*	2 (4.5%) 2;0;0	1 (3.1%) 1;0;0	0 (0%) 0;0;0	2 (13.4%) 1;1;0	4 (5.6%) 3;1;0	18 (42.8%) 15;1;2
Atrophic body gastritis (ABG)						
	1 (2.3%)	8 (25%)	1 (4.5%)	7 (46.7%)	2 (2.8%)	23 (54.7%)

* Number of subjects with different severity of active inflammation, chronic inflammation, gland atrophy, intestinal metaplasia, and ECL cell hyperplasia (eg, 1;0;1 indicates 1 subject with grade 1[mild], 0 with grade 2[moderate], and 1 with grade 3[severe]).

As shown in **Figure 7.1**, among the three subgroups, subjects with atrophic body gastritis yielded significantly higher serum levels of IL-1 β (**A**), IL-6 (**B**), and especially IL-8 (**C**) than those without atrophic body gastritis. Additionally, as shown in **Table 7.2**, subjects with atrophic body gastritis had higher serum gastrin, serum CgA, and serum pepsinogens A and C levels than those without body mucosal atrophy. Conversely, serum pepsinogen A:C ratios were significantly lower in subjects with than in those without atrophic body gastritis.

Figure 7.1. Serum IL-1 β (**A**), IL-6 (**B**), and IL-8 (**C**) levels in subjects with and without atrophic body gastritis (ABG). The 50th percentile (median) is indicated with a line in the box, the end of the box indicates the 25th and 75th percentile, and the 10th and 90th percentile are indicated with error bars. The open circles indicate outliers, which are not extreme values. *P<0.05 and **P<0.01 and ***P<0.0001 vs subjects without atrophic body gastritis

Table 7.2. Serum gastrin, chromogranin A (CgA), pepsinogens A, C, and A:C ratios in patients with and without atrophic body gastritis (ABG).

Serological parameter	Controls		H ₂ RA group		PPI group	
	ABG (-) (n=67)	ABG (+) (n=9)	ABG (-) (n=29)	ABG (+) (n=8)	ABG (-) (n=88)	ABG (+) (n=25)
Gastrin (ng/l)	28.0 (10-73)	87.0 (15-135)†	45.0 (15-88)	65.0 (25-124)*	84.0 (30-258)	140.0 (49-291)†
CgA (nmol/l)	1.8 (1.3-3)	2.3 (1.5-3.5)*	2.0 (1.7-3.2)	2.5 (1.5-3.9)	2.8 (1.8-8.5)	5.4 (1.8-19.5)†
Pepsinogen A (µg/l)	32.0 (21-52)	36.0 (15-50)	37.0 (14-66)	46.0 (35-56)	72.0 (34-151)	108.0 (60-202)‡
Pepsinogen C (µg/l)	9.0 (5-23)	18.0 (4-29)*	10.0 (4-28)	20.0 (14-26)*	20.0 (10-48)	56.0 (30-90)‡
Pepsinogen A:C ratio	3.3 (2.1-5.5)	1.9 (1.4-3.6)†	3.3 (2.1-5)	2.0 (1.7-3.3)†	3.5 (2.1-5.1)	2.1 (1.7-2.7)‡

Data expressed as median values (interquartile ranges).

*P<0.05; †P<0.01; ‡P<0.0001 vs patients without atrophic body gastritis, Mann-Whitney U test.

In the entire series, positive correlations were found between serum gastrin and serum IL-1 β ($r=0.38$, $P<0.001$), IL-6 ($r=0.42$, $P<0.001$), and IL-8 ($r=0.50$, $P<0.001$) levels. Conversely, negative correlations were found between serum pepsinogen A:C ratios, and serum IL-1 β ($r= -0.49$, $P<0.001$), IL-6 ($r= -0.45$, $P<0.0001$), and especially IL-8 ($r= -0.65$, $P<0.0001$) (**Figure 7.2**). Additionally, serum CgA positively correlated with serum cytokine levels ($P<0.01$ for all three cytokines). Furthermore, the histological degree of atrophic body gastritis correlated positively to serum gastrin ($r=0.62$, $P<0.001$), CgA ($r=0.50$, $P<0.001$) and IL-8 ($r=0.52$, $P<0.001$) levels, and correlated negatively to serum pepsinogens A:C ratios ($r= -0.69$, $P<0.0001$).

Figure 7.2. Correlation between serum IL-8 levels and serum pepsinogen A:C ratios in 226 subjects ($r= -0.65$, $P<0.0001$).

Among the three pro-inflammatory cytokines, serum IL-8 correlated best with the presence and histological degree of body mucosal atrophy. As such, in an attempt to optimize the diagnostic accuracy for atrophic body gastritis, we have defined the **ABG index**, calculated as the ratio between IL-8 and pepsinogen A:C.

Figure 7.3 illustrates the ROC curves for gastrin alone, pepsinogen A:C ratio alone, combined gastrin and pepsinogen A:C ratio, and finally for the **ABG index**, in the prediction of atrophic body gastritis. The values of the AUCs ($\pm$ SE) corresponding to each potential predictor of this condition are listed in **Table 7.3**. As serum gastrin and serum pepsinogens could be influenced by concomitant use of acid-

suppressive medication^{17,18,24}, the patients on acid suppression and the non-treated controls were analysed separately. However, the distribution of the ROC curves was similar in patients on acid suppression and in non-treated controls (**Table 7.3**). Thus, combining all subjects, we found that **ABG index** displayed the greatest AUC (0.910 ± 0.029); this was significantly greater than that of serum gastrin alone (0.721 ± 0.042 , $P=0.018$), serum pepsinogen A:C ratio alone (0.830 ± 0.031 , $P=0.049$), and also than the combined use of gastrin and pepsinogen A:C ratio (0.806 ± 0.035 , $P=0.046$).

Figure 7.3 Receiver operating characteristics (ROC) curves for the prediction of atrophic body gastritis in 226 subjects. The area under the curve (AUC) for the **ABG-index** was significantly greater than that of serum gastrin alone ($P=0.018$), serum pepsinogen A:C ratio ($P=0.049$), and than the combined use of serum gastrin and pepsinogen A:C ratio ($P=0.046$).

Table 7.3. The areas under the curves (AUC) and the standard errors (SE) for serum markers of atrophic body gastritis.

Serum marker	AUC ($\pm$ SE)		
	All (n=226)	Controls (n=76)	Patients on acid suppression (n=150)
Gastrin (ng/l)	0.721 ± 0.042	0.690 ± 0.072	0.734 ± 0.041
Pepsinogen A:C ratio	0.830 ± 0.031	0.802 ± 0.092	0.867 ± 0.020
Combined gastrin and pepsinogen A:C ratio	0.806 ± 0.035	0.797 ± 0.091	0.820 ± 0.028
ABG index (pg/ml)	$0.910 \pm 0.029^*$	0.863 ± 0.072	0.942 ± 0.021

*The AUC for the **ABG index** was greater than for gastrin ($P=0.018$), pepsinogen A:C ratio ($P=0.049$), and the combined gastrin and pepsinogen A:C ratio ($P=0.046$).

Table 7.4 shows the best cut-off values and the corresponding performance characteristics for the above-mentioned tests. The best cut-off values for detection of atrophic body gastritis were: 116 ng/L, for serum gastrin; 2.5, for serum pepsinogen A:C ratio; and 1.8 pg/ml, for the **ABG index**. Using serum gastrin (cut-off value: 116 ng/L), 179 out of 226 (79%) cases were classified correctly. The corresponding figure for serum pepsinogen A:C ratio (cut-off value: 2.5) was 200 out of 226 (88%) cases. The best test accuracy was achieved using the **ABG index** (cut-off value: 1.8 pg/ml): 205 out of 226 (91%) cases were classified correctly. In the remaining 21 (9%) cases, discrepancy was found between the biochemical (**ABG index**) and histological evidence of atrophic body gastritis. Among the 11 (5%) cases with a negative biochemical test and positive histology, all but one displayed chronic inflammation of the body mucosa (3 mild, 5 moderate, and 3 severe); additionally, 8 displayed gland atrophy (8 mild, and only 2 moderate). Among the 10 (4%) cases with a positive biochemical test and negative histology, all displayed chronic inflammation of the body mucosa (1 mild, 5 moderate, and 4 severe).

Table 7.4. The cut-off values and test performance characteristics for serum gastrin, pepsinogen A:C ratio, and the **ABG index** in the detection of atrophic body gastritis.

Serum marker	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Gastrin (ng/l)	116	60	83	46	90	79
Pepsinogen A:C ratio	2.5	62	95	72	92	88
ABG index (pg/ml)	1.8	74	94	76	94	91

Discussion

This study confirms previously reported data showing that a high serum gastrin and a low pepsinogen A:C ratio are good predictors for the presence and severity of atrophic body gastritis^{9,11,13-15}. It is, however, the first study to indicate that the measurement of serum pro-inflammatory cytokines may provide additional information on the histomorphologic status of the gastric body mucosa, as compared to the aforementioned biochemical tests. We have shown that the simultaneous presence of a high IL-8 level and a low pepsinogen A:C ratio - defined by us as the **ABG index** - yielded the best test accuracy in predicting atrophic body gastritis. At a cut-off value of 1.8 pg/ml, the proportion of well-predicted cases was 91%. Importantly, as illustrated by the ROC curves, the **ABG index** performed significantly better than serum gastrin alone, serum pepsinogen A:C ratio alone, and than the

combined use of gastrin and pepsinogen A:C ratio. The calculation of such index is therefore appealing for several reasons:

1. From a **diagnostic** viewpoint, a simple and reliable test to detect atrophic body gastritis may be useful in a community setting, for screening populations exposed to risk factors for a more rapid development of atrophic gastritis. In recent years, much attention and research effort have emerged to identify these risk groups. As such, compelling evidence now indicates that first-degree relatives of patients with gastric cancer carry an increased risk of developing atrophic body gastritis in the presence of *H.pylori* infection³³; genetic traits, such as polymorphisms of the genes encoding IL-1 β probably underlie such development³⁴. Also, long-standing acid suppressive-therapy in *H.pylori*-positive subjects increases the risk of atrophic body gastritis³⁵⁻³⁸; the simultaneous gastric infection with non-*H.pylori* bacterial species may be a co-factor in this process^{19,39,40}. Whatever the risk factors would be, in these subjects, serological testing for atrophic body gastritis may be an aid in clinical decision making, orientating further referral for endoscopic and histologic examination.
2. From a **scientific** viewpoint, the **ABG index** provides a better insight on the morphologic status of the body mucosa. In interpreting its significance, it should be borne in mind that a low pepsinogen A:C ratio reflects 'established' glandular loss in the body mucosa, whilst a simultaneous high IL-8 level more probably reflects *ongoing chronic active inflammation*, which in long-term may facilitate the development of gastric cancer.

Several studies addressed the issue of serologic screening for atrophic body gastritis, as this condition carries an increased risk for the development of gastric cancer^{7,8}. In an earlier report of 171 first-degree relatives of patients with pernicious anaemia, Varis *et al.*⁹ found that a high serum gastrin and a low serum pepsinogen A were equally specific (97%) for severe atrophic body gastritis, but the sensitivity of pepsinogen A was greater than that of gastrin (91% vs 83%). Furthermore, they found a specificity of 100% for the combined use of gastrin and pepsinogen A. In a study looking at the prevalence of atrophic gastritis in 207 consecutive Scandinavian patients, Oksanen *et al.*⁴¹ found test specificities for serum gastrin (cut-off >111 pg/ml) of 96%, and for serum pepsinogen A (cut-off < 28 μ g/L) of 99%, whilst the test sensitivities were 75% and 81%, respectively. In a recent literature survey comparing the test characteristics of various screening methods for atrophic body gastritis, Ley *et al.*⁴² observed a wide variation in this

respect. They suggested that this could be due to differences in populations studied (e.g. in selection criteria, nature and array of the underlying risk factors, as well as dissent on definition of atrophic gastritis, and very different cut-off values of the tests applied. In a clinical trial conducted by their own group in Chiapas (Mexico) on 205 healthy volunteers aged 40+ years, they found that serum pepsinogen A:C ratio (cut-off <2.5) had a good specificity (95.7%), but a poor sensitivity (13.7%) for the detection of body atrophy⁴².

Taken together, these findings and our own data indicate that, in general, serum markers of atrophic body gastritis are useful for 'ruling out' this condition (good negative predictive value), although their value for 'ruling in' the diagnosis is merely moderate (moderate positive predictive value). This observation is probably not surprising and two eventually confounding situations will be further discussed: First, a positive test is more likely to occur in the presence of severe atrophy; hence, a negative biochemical test may simply imply less advanced atrophic changes. Second, it is known that in the presence of chronic active inflammation, the detection and gradation of gland atrophy may be overestimated^{43,44}; hence, signs of atrophy will be detected in spite of a negative biochemical test. Such explanations could account for the 5% of the cases in our series, having a 'normal **ABG index**', despite positive histology. Conversely, 4% of the cases had a high **ABG index**, despite negative histology. A possible explanation for this latter discrepancy is that, chronic inflammation involves pro-inflammatory cytokines at an *early stage*; this cytokine activation may then result in progression of inflammation to gland atrophy, and eventually changes in serum gastrin and pepsinogens profiles. In this sense, a clear advantage emerges from using the **ABG index**: the likelihood to detect *early* (pre-atrophic) changes of the gastric body mucosa.

In summary, this study has shown that the measurement of serum pro-inflammatory cytokines, especially IL-8, provides insights on the histomorphologic status of the gastric body mucosa, being a useful complement to the known predictors, gastrin and pepsinogen A:C ratio. Furthermore, the **ABG index** was a better predictor of atrophic body gastritis than serum gastrin and serum pepsinogen A:C ratio alone. We therefore recommend the **ABG index** as a reliable, non-invasive screening test for atrophic body gastritis in large-scale population studies.

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Chapter **8**

General discussion

General discussion

Recent years have witnessed widespread use of acid-suppressive medication in the management of various acid-related gastrointestinal disorders. In the Netherlands, 1-2% of the general population uses maintenance therapy with proton pump inhibitors¹. This is not surprising, considering that the PPIs have the best profile of efficacy and adverse events. As a result of the huge experience with these pharmacological agents, many issues centred around their potential side-effects have been presently answered. It is important to realise, however, that there is a limited understanding of the complex changes in intragastric environment during acid suppression. Such insights are needed, since they may be essential for patient safety and further drug development.

The gist of this thesis focused on the development of chronic atrophic gastritis and ECL cell hyperplasia during treatment with acid-suppressive medication. Particular attention was paid to the role of intragastric *H.pylori* and non-*H.pylori* bacteria in this context. Finally, the clinical utility of serological markers for the aforementioned conditions was investigated.

Gastrin and enterochromaffin-like cell proliferation during acid suppression

Hypergastrinaemia and the proliferation of gastric ECL cells are common in *H.pylori*-infected subjects treated long-term with acid suppression. So far, the alleged risk of developing gastric carcinoid tumors in this setting has not been substantiated². However, the consequences of 'life-long' iatrogenic hypergastrinaemia on the ECL cells in humans are presently not known, favouring a prudent attitude in this respect. As such, the non-invasive screening for ECL cell hyperplasia may be advisable in young GORD patients who, will not chose for antireflux-surgery, but instead will undergo long-term treatment with acid inhibitory medication³. Recently, Waldum *et al.* described elevated serum chromogranin A after short-term treatment with omeprazole, and proposed its use as a marker of ECL cell hyperactivity and/or hyperplasia⁴. In this thesis, we have expanded these observations by showing that *long-term* treatment with PPIs was particularly associated with elevated serum CgA, compared to H₂RA treatment and non-treated subjects (**Chapter 3**). Novel aspects emerged regarding the relationship between serum CgA levels and *H.pylori*-induced chronic gastritis during treatment with acid-suppressive medication (**Chapter 4**). *H.pylori*-positive subjects treated with PPIs

displayed markedly elevated serum CgA, reflecting more severe hyperplastic lesions of the ECL cells. Interestingly, the correlation between gastrin and CgA seemed to be fitted best by a quadratic function. In contrast to serum gastrin - which described a 'plateau phenomenon' - serum CgA levels gradually increased during long-term acid suppression and were paralleled by increasing prevalence of ECL cell hyperplasia. In view of these observations, we suggested that increased serum CgA during acid-suppressive therapy initially may reflect a *hypersecretory state* of the ECL cells, in response to sustained hypergastrinaemia, whilst during long-term acid suppression such increase is likely to be the result of ECL cell *hyperplasia*. From a clinical viewpoint, serum CgA was found to be a useful test in screening for ECL cell hyperplasia in this setting. Furthermore, we suggested a potential benefit for serum CgA, compared to serum gastrin, as this test was equally sensitive but more *specific* than serum gastrin.

Non-*H.pylori* bacteria during acid suppression

The concept of 'intra-gastric bacteria' ranks, undoubtedly, among the most fascinating topics in the history of gastroenterology. In 1875, the old dictum that no bacteria could survive in the acidic milieu of the stomach faded when Bottcher & Letulle demonstrated bacterial colonies in the mucosal margins of ulcers⁵. They suggested a causative bacterial agent in ulcer disease. In 1889, Jaworski was the first who described spiral microorganisms (*Vibrio rugula*) in the gastric contents from humans⁵. Bacteriological examinations then revealed a wide spectrum of intra-gastric microorganisms, such as *Streptococci*, *Staphylococci*, and bacilli, albeit of unknown significance for gastric pathology. It was about one century latter (1984), when Warren & Marshal demonstrated that an infective agent, *Campylobacter pyloridis* (later named *Helicobacter pylori*) was responsible for gastric and duodenal (ulcer) disease⁶. This was the beginning of a tremendously rapid growing area, and *H.pylori* has polarised this scientific scene for more than two decades. By contrast, the earlier identified, other 'non-*H.pylori* bacterial species' acted an obscure role only. In 1975, Correa *et al.* proposed a model for the histopathogenesis of gastric cancer of the intestinal type⁷. This hypothesis was further substantiated by Ruddell *et al.*^{8,9}, who showed that chronic hypochlorhydria (e.g., due to atrophic gastritis with/without pernicious anaemia or post-gastrectomy) promotes the overgrowth of intra-gastric nitrate-reducing bacteria, which in turn may lead to an increased production of carcinogenic NOCs. These could initiate a series of mutations resulting in intestinal metaplasia, dysplasia, and finally gastric carcinoma. It has been argued, however, that given the technical difficulties in measuring NOCs, as well as the lagtime

between the aforementioned events and development of gastric carcinoma it must be difficult to confirm or refute such theory¹⁰.

After the introduction of potent inhibitors of gastric acid secretion, much scientific interest has been directed to the intragastric growth of non-*H.pylori* bacteria¹¹; thus far the clinical relevance of this phenomenon remains controversial. In line with previous studies by our group (in subjects with low-acid states of different origin)^{12,13}, in this thesis we have shown that non-*H.pylori* bacteria not only contaminate the gastric juice but also colonise the gastric mucosa of the majority of patients treated long-term with acid suppression (**Chapter 5**). Culture of gastric juice identified mainly bacteria of oropharyngeal origin, although faecal-type flora was also isolated in some cases. Comparison between gastric luminal and mucosal growth of non-*H.pylori* bacteria revealed that the latter could provide important information concerning the changes in intragastric environment under these conditions. Thus, treatment with PPIs resulted in significantly higher pH levels and higher prevalence of non-*H.pylori* bacteria in gastric juice than the treatment with H₂RAs. Surprisingly, in spite of these differences in gastric juice bacterial overgrowth, the PPI-treated subjects and the H₂RA-treated subjects did not differ with regard to non-*H.pylori* bacterial growth in the gastric mucosa ($\pm 60\%$ in both groups). A plausible interpretation would be that the gastric luminal bacterial growth is a pH-dependent phenomenon, whilst the gastric mucosa during acid suppression seems to behave as a constant 'bacterial reservoir'.

Remarkably, in most cases non-*H.pylori* bacteria were located superficially, in the gastric mucus layer, but occasionally they *co-located* with *H.pylori*, on deeper mucosal positions (*i.e.*, gastric crypts) (**Chapter 5**). With these observations in mind, we next addressed the relationship between gastric mucosal *H.pylori*, non-*H.pylori* bacteria and chronic gastritis during acid-suppressive therapy.

In a larger context, it is worthwhile to mention that, as of today, increasing evidence supports a role of commensal bacteria in several models of compromised gut (such as in patients with inflammatory bowel disease or celiac disease)¹⁴. In view of this, to argue that intragastric non-*H.pylori* bacteria (secondary to acid suppression) is an 'innocent bystander' only is a dogmatic viewpoint: it should be pointed out that such bacteria represent, *de facto*, the *predominant* intragastric flora during acid suppression, which shares the habitat with the recognised gastric pathogen (*H.pylori*), and could yield complex, largely unidentified pathogenicity factors.

In this thesis we presented 'back-to-back' arguments that the gastric *double infection* with *H.pylori* and non-*H.pylori* bacteria during acid suppression may play a role in the aggravation of chronic gastritis and accelerated development of atrophy. *H.pylori*-positive subjects treated

long-term with acid suppression were characterised by three features: non-*H.pylori* bacterial growth in the gastric mucosa, increased risk for atrophic body gastritis and increased serum pro-inflammatory cytokine levels, particularly IL-1 β and IL-8. This was in stark contrast to the *H.pylori*-negative subjects on long-term PPI treatment, who had a very low rate of atrophy. The simultaneous gastric infection with *H.pylori* and non-*H.pylori* bacteria was associated with the highest serum levels of pro-inflammatory cytokines and the highest risk for atrophic gastritis. The type of interaction between *H.pylori* and non-*H.pylori* bacteria appeared to be synergistic. Additionally, we found that *H.pylori* *cagA*-positivity increased the risk of atrophy, particularly of the antral mucosa. Taken together, these results suggest that on a background of compromised gastric mucosal barrier (caused by the *H.pylori* infection), the co-infection with non-*H.pylori* bacterial strains may upregulate the pro-inflammatory cytokine response, thereby leading to aggravation of the chronic gastritis and a more rapid development of atrophy (**Chapter 6**). However, further investigation is needed to elucidate the cellular and molecular mechanisms by which a double gastric infection may contribute to the pathogenesis of chronic atrophic gastritis.

The strength of these data resides in the large study material (including a sizeable control group). Furthermore, in an attempt to reflect an everyday clinical situation, patients were recruited consecutively via an open-access endoscopy unit. Every effort was made to estimate the role of the time factor in the development of chronic atrophic gastritis. However, data were gathered in a cross-sectional design. Hence, the question as to whether non-*H.pylori* bacterial flora is a *causal risk factor* for the development of atrophic gastritis or merely a *marker* for a risk condition cannot be finally answered and remains open for future research.

In this sense, it would be important to further identify these bacteria through culture and/or molecular methods, to unravel their potential pathogenicity factors (e.g., adhesion properties, production of cytotoxic and pro-inflammatory agents), as well as their potential role in the modulation of immune response. It is worth commenting that we found a weak association between presence of non-*H.pylori* bacteria alone, serum pro-inflammatory cytokines, and the risk for atrophy (**Chapter 6**), with numerically higher cytokine levels in these subjects (especially IL-8) and a trend towards increased risk for body atrophy compared to subjects without any type of bacteria. Importantly, recent evidence from animal studies indicates that *concurrent* infections may alter the Th1-polarised cytokine response associated to the *H.pylori* infection¹⁵. The behaviour of Th1/Th2 balance during the gastric infection with *H.pylori* and other non-*H.pylori* bacteria was not addressed in these studies. It will be, therefore, the challenge of future research to define the

peripheral and gastric mucosal T cell phenotype in relation to **total** intragastric bacteria. Furthermore, longitudinal studies are needed to elucidate the *time-course* of gastric mucosal changes due to a double bacterial infection.

Atrophic body gastritis and intestinal metaplasia are considered preneoplastic gastric lesions^{16,17}, and therefore efficient screening for such lesions is an important public health issue. We have investigated whether the measurement of serum pro-inflammatory cytokines in this context offers additional information on the histomorphologic status of the body mucosa, compared to the measurement of serum gastrin and serum pepsinogens (**Chapter 7**). In an attempt to optimise the prediction of body atrophy, we have defined the atrophic body gastritis (**ABG**) *index*, that is the ratio between the simultaneously measured IL-8 level and pepsinogen A:C. The **ABG index** performed significantly better than serum gastrin alone and serum pepsinogen A:C alone; at a cut-off value of 1.8 pg/ml the total proportion of well-predicted cases was 91%. It is conceivable that besides indicating 'established' glandular loss in the body mucosa, this test may reflect *ongoing chronic active inflammation*, which in long-term may progress to gastric cancer.

The results presented in this thesis add to a growing body of evidence, indicating that chronic atrophic gastritis is a multifactorial condition. They also provide compelling arguments that, apart from the genetic set-up and *H.pylori*-related factors, the environmental factors, like chronic use of acid-suppressive medication, may play an important role in this development. **Figure 8.1** summarizes the available data concerning the pathophysiology of chronic atrophic gastritis: chronic hypochlorhydria and subsequent overgrowth of non-*H.pylori* bacteria may enhance and perpetuate the gastric mucosal damage initiated by the *H.pylori* infection through: (i) increased *N*-nitrosation¹⁸, (ii) bacterial production of acetaldehyde¹⁹, and (iii) upregulation of the pro-inflammatory cytokines²⁰.

What are the clinical implications of these studies?

*Given the potential harmful effect of the double gastric infection with *H.pylori* and non-*H.pylori* bacteria - namely worsening of body gastritis and an accelerated development of body atrophy with hyperplasia of the ECL cells - one should consider a strategy of testing for *H.pylori* followed by eradication therapy when positive, before starting long-term gastric acid inhibition.*

Figure 8.1 Schematic diagram illustrating possible key events in the development of corpus atrophic gastritis.

The most important argument in favour of such strategy is the potential reduction in chronic body gastritis and development of atrophy^{21,22}, which is a precursor lesion for non-cardia gastric cancer. The arguments against this strategy include the cost, the potential adverse events associated to antibiotic therapy, and potential negative consequences of eradication, such as increasing incidence of GORD²³. Pending available evidence, the recent European Consensus regarding *H.pylori* management guidelines (Maastricht, 2000)²⁴ stated that: '*H.pylori* should be eradicated in patients requiring long-term profound acid suppression'. The present studies will probably increase the level of scientific evidence in favour of this clinical policy.

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Chapter 9

Summary and concluding remarks

Summary

This thesis enlightens some aspects concerning the development of chronic atrophic gastritis and ECL cell hyperplasia during acid-suppressive therapy. Special attention was dedicated to the role of intragastric *H.pylori* and non-*H.pylori* bacteria in this context. Finally, the clinical utility of serological markers for these conditions was addressed.

In **Chapter 1**, an overview is presented of current knowledge about the changes in gastric mucosa and luminal environment during treatment with acid-suppressive medication. In summary, worldwide experience with these pharmacological agents presently reassures their general safety. However, the mechanistic aspects underlying their gastrointestinal effects are still far from complete understanding. Such information is needed, as this may have impact on patient care and further drug development.

Chapter 2 presents the aims and outline of the thesis.

In **Chapter 3** the relationship between serum gastrin and serum CgA levels during medium- and long-term acid-suppressive therapy was investigated. We reported significantly higher serum gastrin and serum CgA levels after long-term PPI therapy compared to H₂RA therapy and non-treated controls. Elevated serum CgA correlated to positive *H.pylori* serology and the length of acid inhibitory treatment. We next sought to determine the relationship between serum CgA levels and fundic ECL cell hyperplasia during acid suppression (**Chapter 4**). *H.pylori*-positive subjects treated long-term with PPIs had markedly elevated serum CgA, which correlated to the severity of ECL cell hyperplasia. The clinical utility of serum CgA in this regard was compared to that of serum gastrin. As such, we found that during long-term acid suppression, serum CgA was equally sensitive but more *specific* than serum gastrin for the detection of ECL cell hyperplasia. In view of the potential negative consequences of iatrogenic hypergastrinaemia on gastric mucosa, it might be prudent to monitor serum CgA levels during long-term acid suppression. This holds especially true in young GORD patients, not referred for antireflux-surgery.

Chapter 5 describes the gastric *luminal* and *mucosal* growth of non-*H.pylori* bacteria during acid suppression. In gastric juice, prevalence rate for non-*H.pylori* bacteria was higher in the PPI group than controls and H₂RA group; no difference was found between the H₂RA group and controls. In gastric mucosa, prevalence rates for non-*H.pylori* bacteria were higher in patients on acid suppression - either with PPIs or H₂RAs - than in the controls; no difference was found between the PPI group and H₂RA group. Both luminal and mucosal growth of non-*H.pylori*

bacteria were significantly greater in *H.pylori*-positive than -negative patients on PPIs. Luminal growth of non-*H.pylori* flora increased with the intragastric pH level, while mucosal bacterial growth increased with the duration of acid inhibition.

In **Chapter 6** the relationship between intragastric bacteria and chronic atrophic gastritis during acid-suppressive therapy has been studied. The hypothesis tested was that the *gastric double infection* with *H.pylori* and non-*H.pylori* bacteria may bring about an enhanced pro-inflammatory cytokine response, thereby contributing to the accelerated development of atrophic gastritis under these conditions. We reported that the simultaneous presence of *H.pylori* and non-*H.pylori* bacteria was associated with elevated serum pro-inflammatory cytokines, particularly IL-1 β and IL-8, and a markedly increased risk for atrophic gastritis. The type of interaction between *H.pylori* and non-*H.pylori* bacteria was *synergistic*. In view of these findings, we suggested that non-*H.pylori* bacteria may be a *co-factor* in the development of atrophic gastritis during acid-suppressive therapy. Further studies are needed, however, to elucidate whether this association reflects a causative role.

Chapter 7 addressed the clinical utility of measuring serum pro-inflammatory cytokines - IL-1 β , IL-6, and IL-8 - together with serum gastrin and serum pepsinogens in screening for atrophic body gastritis. In an attempt to optimise the detection of this condition we have defined the atrophic body gastritis (**ABG**) *index*, as the ratio between the simultaneously measured IL-8 level and pepsinogen A:C. The **ABG index** performed significantly better than serum gastrin alone and serum pepsinogen A:C alone. Additionally, such test seems to reflect *ongoing chronic active inflammation*, which in long-term may evolve to gastric cancer. We therefore recommend its use in screening for atrophic body gastritis in large-scale population studies.

Finally, **Chapter 8** synthesises the results of these studies, and indicates their possible implications for clinical practice and future research into this area.

What do these studies add to current knowledge?

- *Long-term acid suppression in the presence of *H.pylori* infection is associated with:*
 - *non-*H.pylori* bacterial growth in the gastric mucosa*
 - *increased serum pro-inflammatory cytokine levels*
 - *high rate of atrophic body gastritis and ECL cell hyperplasia.*
 - *The gastric double infection with *H.pylori* and non-*H.pylori* bacteria increases the risk of atrophic body gastritis, possibly through eliciting an enhanced pro-inflammatory cytokine response. In view of this, *H.pylori* eradication should be recommended before the start of long-term acid suppression in order to prevent the potential cumulative damage to the mucosa due to a double bacterial infection.*
 - *The ABG index is a reliable screening test for atrophic body gastritis. Additionally, serum CgA is a useful screening test for ECL cell hyperplasia, being more specific than serum gastrin in this regard.*
-

In conclusion, the research described in this thesis may be relevant to our evolving understanding of chronic atrophic gastritis as a multifactorial process. The host genetic factors set up the baseline immune response to the *H.pylori* infection. Additionally, the *environmental factors* might play a major role in the modulation of this response, and the final determination of disease outcome.

It is probably too early to draw firm conclusions about the interrelation between *H.pylori* and non-*H.pylori* bacteria in the pathogenesis of chronic atrophic gastritis and gastric cancer. As a first step in this sense, efforts should be directed to identify the **total** intragastric bacteria in the context of acid suppression, their pathogenicity factors, as well as their potential role in gastric immune response.

Finally, we have gathered now substantial evidence to advocate for the eradication of *H.pylori* in patients considered for long-term acid-suppressive therapy.

Chapter **10**

Samenvatting & conclusies

Samenvatting

In dit proefschrift worden verscheidene aspecten betreffende de ontwikkeling van chronische atrofische gastritis en hyperplasie van de ECL cellen tijdens de behandeling met maagzuurremmende medicatie beschreven. Bij deze ontwikkeling wordt er speciale aandacht besteed aan de rol van *H.pylori* en non-*H.pylori* maagbacteriën. Tevens is de klinische bruikbaarheid van serologische markers voor chronische atrofische gastritis en voor hyperplasie van de ECL cellen bepaald.

Het bestudeerde cohort bestond uit 230 opeenvolgende dyspeptische patiënten die een gastroscopie ondergingen in het AZM. Hierbij werden drie groepen onderscheiden: patiënten met gastro-oesofageale reflux ziektes die werden behandeld met proton pomp inhibitoren (PPI, n=117) of met histamine₂-receptor antagonisten (H₂RA, n=37) en niet behandelde controle patiënten, met normale endoscopische bevindingen (n=76).

In **Hoofdstuk 1** wordt een overzicht gegeven van de actuele kennis ten aanzien van de veranderingen in het maagmilieu tijdens de behandeling met maagzuurremmers, in het bijzonder de *hypergastrinaemie, de proliferatie van ECL cellen en intragastrische bacteriële overgroei*. De pathofysiologie van bovengenoemde veranderingen is voornamelijk echter weinig bekend. Kennis omtrent deze aspecten is essentieel voor de klinische behandelingsstrategieën en toekomstige ontwikkelingen van de maagzuurremmende medicatie.

In **Hoofdstuk 2** wordt de achtergrond van dit proefschrift en de daaruit voortvloeiende vragen besproken.

In **Hoofdstuk 3** wordt de relatie bestudeerd tussen serum gastrine en serum chromogranine A (CgA), een marker voor neuroendocrine proliferatie, tijdens middellange en langdurige maagzuurremming, om na te gaan of deze behandelingen in staat zijn ECL hyperactiviteit of ECL cell hyperplasie te induceren.

Serum gastrine en serum CgA waarden waren significant hoger bij patiënten die behandeld werden met een PPI ten opzichte van H₂RA-behandelde en de niet-behandelde controles. Er werden positieve correlaties gevonden tussen serum CgA waarden en een positieve serologie voor *H.pylori* en tussen serum CgA en de duur van de behandeling met een PPI.

In **Hoofdstuk 4** wordt de relatie onderzocht tussen serum gastrine, serum CgA waarden en hyperplasie van de ECL cellen in de mucosa van de maagcorpus/fundus tijdens maagzuurremmende therapie. Hierbij werd aangetoond dat *H.pylori*-positieve patiënten met langdurige PPI behandeling worden gekenmerkt door een toename in serum CgA waarden. Deze verhoogde waarden zijn gecorreleerd met de aanwezigheid en de histologische graad van de hyperplasie van de ECL cellen. Daarnaast is de klinische bruikbaarheid van serum CgA als marker voor de ECL cel hyperplasie vergeleken met serum gastrine: serum CgA was even sensitief (91.3%), maar had een hogere specificiteit dan serum gastrine (73% vs 43%, $P < 0.0001$) voor de detectie van hyperplasie van de ECL cellen bij patiënten die langdurig behandeld zijn met maagzuurremmers.

Vanwege het potentiële negatieve effect van de iatrogene hypergastrinaemie (b.v. inductie van neoplasie in de maag en elders), is het wenselijk om de CgA waarden bij deze patiënten te monitoren. Dit geldt vooral voor jonge patiënten met gastro-esofageale reflux ziektes die langdurig behandeld moeten worden met maagzuurremmers.

De intragastrische bacteriële flora tijdens zuurremmende therapie wordt beschreven in **Hoofdstuk 5**. Hiervoor is de overgroei van non-*H.pylori* bacteriën in het *maagsap* en in de *mucosa* van de maag bestudeerd. Er werd aangetoond dat de PPI groep een significant hogere prevalentie heeft van non-*H.pylori* bacteriën in het *maagsap* ten opzichte van de H₂RA groep en de controles (58.7% vs 22.6% en vs 30.6%). Daarnaast werd aangetoond dat zowel de PPI groep als ook de H₂RA groep een significant hogere prevalentie heeft van de non-*H.pylori* bacteriën in de *mucosa* dan de controles (61.1% en 59% vs 29%). Zoals werd verwacht was de overgroei van de non-*H.pylori* bacteriën in het maagsap positief gecorreleerd met de intragastrische pH-waarde. Het was echter opmerkelijk dat de overgroei van de non-*H.pylori* flora in de mucosa steeg met de duur van de behandeling en dat non-*H.pylori* bacteriën significant vaker werden aangetoond bij de *H.pylori*-positieve patiënten dan bij de *H.pylori*-negatieve patiënten. Het kan verondersteld worden dat de maagmucosa een 'reservoir' vormt voor non-*H.pylori* bacteriën tijdens krachtige en langdurige maagzuurremming.

In **Hoofdstuk 6** wordt de nadruk gelegd op de relatie tussen de intragastrische bacteriële flora en de ontwikkeling van chronische (atrofische) gastritis tijdens maagzuurremmende therapie. Het is bekend dat bij *H.pylori* positieve patiënten langdurige behandeling met maagzuurremmende medicatie geassocieerd is met een toename van corpus gastritis en een versnelde ontwikkeling van chronische

atrofische gastritis. Het pathofysiologisch mechanisme dat hieraan ten grondslag ligt is tot op heden onbekend.

De hypothese van de in dit proefschrift gepresenteerde studie was dat een *dubbele infectie* van de maag met *H.pylori* én non-*H.pylori* bacteriën tijdens zuurremmende therapie zou kunnen leiden tot een verhoogde inflammatoire respons, welke aanleiding kan geven tot een versnelde ontwikkeling van atrofische gastritis. Omdat atrofie in de corpus mucosa een precursor laesie is van maagkanker, werd specifiek gekeken naar het vóórkomen hiervan.

Met behulp van een logistische regressie analyse bleek dat *H.pylori* (OR 11.7), non-*H.pylori* bacteriën (OR 6.4) maar met name de gelijktijdige aanwezigheid van beide typen bacteriën (OR 20.4) geassocieerd was met een verhoogd risico voor atrofie in de corpus mucosa. Daarnaast was de gelijktijdige aanwezigheid van *H.pylori* en non-*H.pylori* bacteriën geassocieerd met een sterke toename van de serum pro-inflammatoire cytokines, IL-1 β , IL-6 en IL-8. De interactie tussen *H.pylori* en non-*H.pylori* bacteriën bij deze ontwikkeling was synergistisch. Gebaseerd op deze bevindingen stellen we voor dat de non-*H.pylori* bacteriële flora een *co-factor* is bij de ontwikkeling van atrofische gastritis tijdens maagzuurremming. Longitudinale studies zijn nodig om na te gaan of dit verband causaal is.

Deze studie heeft directe consequenties voor de klinische behandeling van patiënten die in aanmerking komen voor langdurige maagzuurremmende medicatie. Bij deze patiënten is *screening* op de aanwezigheid van *H.pylori* en de *eradicator* hiervan aan te bevelen vóór de start van een langdurige behandeling met maagzuurremmers. Hiermee kan het potentiële schadelijke effect van een *dubbele infectie* van de maag voorkómen worden.

In **Hoofdstuk 7** wordt de klinische bruikbaarheid onderzocht van de bepaling van het profiel van de pro-inflammatoire cytokines, IL-1 β , IL-6 en IL-8, samen met serum gastrine en serum pepsinogeen A en C voor de screening van atrofische corpus gastritis. Hoge serum gastrine waarden en een lage pepsinogeen A:C ratio zijn bekende voorspellers van atrofie in de corpus mucosa. De mogelijke diagnostische waarde hierbij van pro-inflammatoire cytokines is niet bekend. Daarom hebben wij de zogenoemde '*ABG (atrophic body gastritis) index*' voorgesteld en gedefinieerd als de *ratio* tussen serum IL-8 waarde en serum pepsinogeen A:C. Deze *ABG index* blijkt een significant betere voorspeller voor atrofische corpus gastritis te zijn dan serum gastrine en serum pepsinogeen A:C ($P < 0.05$). Bovendien weerspiegelt deze test de '*continue chronische ontsteking*' van de corpus mucosa, welke aanleiding kan geven tot de ontwikkeling van atrofische gastritis en maagkanker. De *ABG index* zou gebruikt kunnen worden bij screening

van 'hoge-risico' populaties als voorspeller voor atrofische gastritis in het corpus.

Hoofdstuk 8 bevat de slotdiscussie en conclusies omtrent de veranderingen in het maagmilieu tijdens middellange en langdurige behandeling met maagzuurremmers, waarbij de gegevens van de klinische studies, gepresenteerd in de voorgaande hoofdstukken, worden geïntegreerd. De klinische implicaties van deze bevindingen worden benadrukt.

Wat voegen de studies toe aan de huidige kennis?

- *H.pylori-positieve patiënten die langdurig maagzuurremmers hebben gebruikt worden gekenmerkt door:*
 - *een non-H.pylori bacteriële groei in de maagmucosa,*
 - *een toename van de pro-inflammatoire cytokines in het serum, en*
 - *een versnelde ontwikkeling van atrofische gastritis in het corpus.*
- *De dubbele infectie van de maag met H.pylori en non-H.pylori bacteriën tijdens zuurremmende therapie is geassocieerd met een verhoogd risico voor atrofie, mogelijk als gevolg van een verhoogde inflammatoire respons.*
Daarom is een H.pylori 'test and treat' strategie geïndiceerd alvorens te starten met langdurige maagzuurremmende therapie, om zo het potentiële schadelijke effect van een dubbele infectie van de maag te voorkómen.
- *De ABG index is een betrouwbare screeningstest voor atrofische gastritis in het corpus van de maag.*
- *Serum CgA is een betrouwbare screeningstest voor de ECL cel hyperplasie tijdens zuurremming en specifiekere dan serum gastrine.*

De in dit proefschrift beschreven resultaten kunnen het inzicht in de pathogenese van chronische atrofische gastritis als precancereuze laesie van de maag en veiligheidsaspecten van de behandeling met maagzuurremmers vergroten. Genetische factoren spelen een belangrijke rol bij de ontwikkeling van chronische atrofische gastritis in de aanwezigheid van *H.pylori*, maar zoals nu blijkt kunnen milieu factoren, als een langdurige behandeling met maagzuurremmers, deze ontwikkeling moduleren en de klinische uitkomst bepalen.

Het is waarschijnlijk te vroeg om definitieve conclusies te trekken betreffende de relatie tussen *H.pylori* en non-*H.pylori* bacteriën bij de ontwikkeling van atrofische gastritis en maagkanker. Een eerste stap

hiertoe zou het identificeren moeten zijn van de *totale* intragastrische flora en vervolgens het bestuderen van de pathogenetische mechanismen en de mogelijke rol van deze bacteriën bij de lokale en systemische immuunrespons.

De in dit proefschrift beschreven studies ondersteunen het praktische concept dat eradicatie van *H.pylori*, alvorens te starten met maagzuurremmende therapie, noodzakelijk is.

Addendum

A

D

B

E

C

F

Different patterns of chronic gastritis. (A) diffuse non-atrophic gastritis of the corpus mucosa; (B) antral predominant atrophic gastritis; (C) corpus predominant atrophic gastritis with intestinal metaplasia (H&E). Different patterns of ECL cell hyperplasia in the oxyntic mucosa of patients under long-term acid-suppressive therapy. (D) diffuse; (E) linear; (F) micronodular growth pattern (CgA immunostaining). Original magnification X 200.

Aknowledgement

Acknowledgement

At the very beginning, it was a white sheet of paper and a puzzling equation with many variables:

I was carefully looking to find the right words, when my Dutch colleagues just told me: 'je moet ergens beginnen' (you should start somewhere). So I did! I was always inspired by George Bernard Shaw's words: 'If you have an apple and I have an apple and if we exchange these apples then we will still each have one apple. But if you have an idea and I have an idea and if we exchange these ideas, then each of us will have two ideas...' An extrapolation of this wisdom to my experience in the last years would be: 'Een proefschrift is altijd teamwork' (A PhD thesis always means teamwork). Not only in the sense of resourceful thinking, but mainly in that of strenuous effort and continuity in achieving a goal.

*I would, therefore, like to express my gratitude to **all** the people who have contributed with their own experience to the writing of this thesis.*

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Publications

Publications

ARTICLES

- S. Sanduleanu, M. Stridsberg, D. Jonkers, W. Hameeteman, I. Biemond, Lundqvist, C. Lamers, R.W. Stockbrugger. "Serum gastrin and chromogranin A levels during medium- and long-term acid suppressive therapy: a case-control study". *Aliment Pharmacol Ther* 1999;13:145-53.
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Curriculum Vitae

Curriculum Vitae

Silvia Sanduleanu was born on June 16, 1965 in Dorohoi, Romania. After finishing the High School of Mathematics and Physics 'Grigore Ghica' in Dorohoi (July 1984), she immediately started studying Medicine at the University of Medicine and Pharmacy from Iasi. She graduated and obtained her medical bachelor degree in 1990 (*Cum Laude*). From 1990 to 1995 she was trained as a resident in Internal Medicine at the University Hospital in Iasi (first rank at final examination). In February 1995 she was registered an internist in Romania, became a physician in charge, and was appointed to assistant professor at this university.

In June 1996 she received an EC grant to study at the Department of Internal Medicine, Division of Gastroenterology of the University Hospital Maastricht. She continued with the research work described in this thesis under the supervision of Prof. Dr. R.W. Stockbrügger. From October 1998 to June 1999 she worked as a resident in Internal Medicine at the University Hospital Maastricht, under the supervision of Prof. Dr. H. Hillen. She will continue further training in Gastroenterology.

Her research interest involves precancerous lesions of the gastrointestinal tract, with focus on chronic atrophic gastritis, gastric ECL hyperplasia, and colorectal polyps.