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GASTROENTEROLOGY

Effects of *cagA*+ and *cagA*– strains of *Helicobacter pylori* on the human gastric mucus layer thickness

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Abstract

Background: Infection with cytotoxin-associated gene A (*cagA*) *Helicobacter pylori* is associated with severe gastric diseases, with contradictory views being expressed concerning the effect of *H. pylori* on the gastric mucus thickness. The aim of the present study was to differentiate between the effect of *cagA*+ and *cagA*– strains on gastric mucus thickness.

Methods: Ninety-nine patients without peptic ulcers who were not on medication were randomly recruited from consecutive endoscopy clinics: six biopsies (five antral, one body) were obtained from each patient. Cryostat sections (18 μm) were cut and stained using the modified periodic acid–Schiff/Alcian blue technique. Mucus thickness was measured using computer-assisted light microscopy. The *H. pylori* status was assessed by histology, *Campylobacter*-like organism (CLO)test and culture, and *cagA*+ status determined by polymerase chain reaction (PCR).

Results: There was no significant difference ($P = 0.784$) in mean mucus thickness between *cagA*+ (52.7 ± 1.2 μm, $n = 10$), *cagA*– (46.6 ± 1.1 μm, $n = 18$) or *H. pylori*-negative patients (51.3 ± 1.1 μm, $n = 30$). In *cagA*– patients, mucus thickness was significantly reduced with increased *H. pylori* colonization density, Spearman (r_s) = –0.805, $P < 0.0001$. In contrast, in *cagA*+ patients there was a weak positive, but not significant, association between mucus thickness and *H. pylori* colonization density, $r_s = 0.333$, $P = 0.381$.

Conclusions: The human gastric mucus thickness is not affected by infection with *cagA*+ or *cagA*– strains of *H. pylori* compared with uninfected. Although a trend of increased mucus thickness with *cagA*+ infection was observed.

Key words: *cagA*+, gastric, *Helicobacter pylori*, mucus, thickness.

INTRODUCTION

Mucus is secreted in the stomach by the surface epithelial cells and exists in three phases: as an adherent mucus gel layer; as pre-secreted mucus stored in intracellular vesicles; and as soluble mucus mixed with the luminal contents.[1] The gastric mucus gel layer is an important first line of defence against acid by maintaining a pH gradient across the mucus layer that is neutral towards the gastric mucosa.[2]

The mucus gel layer provides a protective environment for *Helicobacter pylori* to colonize the gastric epithelium.[3,4] Cytotoxin-associated gene A (*cagA*) is a gene located within a 40-kb chromosomal region of *H. pylori* termed the cag-pathogenecity island (cag-PAI).[5] *Helicobacter pylori* strains containing cag-PAI have been found to be associated with more severe gastric diseases, that is, individuals carrying the *cagA*+ *H. pylori* strains have more severe forms of gastric diseases compared to those with *cagA*– strains.[6–8] The *cagA*+ strains are associated with an increased risk of developing peptic ulcer disease[9] and adenocarcinoma of the distal stomach.[10] However, this association of *cagA*+ strains with severe gastric disease does not occur in all geographic regions.[11–13]

Studies on the effects of *H. pylori* on the gastric mucus thickness have produced contradictory findings ranging from a reduction in mucus thickness to no

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Table 1 Studies on mucus gel thickness from human gastric antral biopsies with conflicting results

Study	No. patients			Mean mucus thickness, μm (SD)	
	Total	HP+	HP–	HP+	HP–
Sarosiek *et al.*[14]	32	17	15	85 (27)	175 (67)
Newton *et al.*[15]	40	20	20	94 (24)	106 (30)

HP+, *Helicobacter pylori* positive; HP–, *H. pylori* negative.

effect on thickness (Table 1).[14,15] Many techniques have been cited in the literature for the measurement of the gastric mucus gel-layer thickness including: Alcian blue binding method;[16] slit-lamp and pachymeter;[17] unfixed sectioning technique;[18] direct light microscopy;[19] electron microscopy;[20] conventional periodic acid–Schiff/Alcian blue (PAS/AB) staining;[21] Modified PAS/AB staining;[22] and *in vivo* technique in rats.[23]

The aim of the present study was to differentiate between the effect of *H. pylori cagA*+ and *cagA*– strains on the gastric mucus gel layer thickness, which could help resolve the controversies reported in the literature.

METHODS

Subjects

One hundred patients were randomly recruited from consecutive endoscopy clinics at three hospitals in Yorkshire in the UK without prior knowledge of their *H. pylori* status. The research has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and had been approved by the Ethics Committees (Leeds Health Authority Clinical Research Ethics Committee and the York Research Ethics Committee). Informed consent was obtained from each patient and all were given information sheets prior to participation in the study. Patients were included in the study if they were not taking acid suppressive drugs, non-steroidal anti-inflammatory drugs (NSAIDs) or receiving treatment for *H. pylori*, but excluded if they were known or found to have peptic ulcer disease or gastric cancer. The presenting symptoms of patients included dyspepsia, heartburn and reflex, abdominal epigastric pain, dysphagia, and follow up for Barrot's esophagus or celiac disease. Following recruitment one patient was subsequently excluded due to the presence of a duodenal ulcer. Of the remaining 99 patients, 42 (42%) were male and 57 (58%) were female with mean ages of 46 ± 11 and 51 ± 15 years, respectively (age range, 17–85 years). Six gastric biopsies (five from antrum and one from body) were taken from each patient: one for measurement of the gastric mucus layer thickness, one for polymerase chain reaction (PCR), one for *H. pylori* culture, one for *Campylobacter*-like organism (CLOtest) and two for histology (antrum and body). *Helicobacter pylori* infection was assessed by histology, culture, PCR and CLOtest: patients were considered *H. pylori*-positive if two of the aforementioned tests were positive.

Polymerase chain reaction

Identification of *H. pylori cagA*+ strain was determined by PCR, directly on biopsy specimens, using two pairs of primers (*cagA*, product size 130 bp; urease C, product size 120 bp) synthesized by Dr F Lewis (University of Leeds). The DNA extraction and PCR were performed using standard protocols.

Helicobacter pylori culture

Helicobacter pylori culture was performed using the methods described by Goodwin[24] and Glupczunski.[25] The bacterial viable count (colonization density) was assessed by direct counting of the bacterial colonies on the culture plates that were inoculated with the serial dilutions prepared from the bacterial homogenates using the method described by Collins and Lyne.[26] The bacterial viable count was expressed in colony-forming unit (CFU)/mg of biopsy weight.

Mucus gel-layer thickness measurement

The biopsy for mucus thickness measurement was sandwiched in pig's liver (1 cm × 1.5 cm) for support, wrapped in aluminum foil and then immediately frozen in liquid nitrogen for transport and stored at –20°C until processed. For each biopsy, thin tissue sections 18 μm thick were cut vertically on the gastric mucosa using a microtome (Leica, model CM 1510-1, Germany). The sections were mounted on slides coated with poly-L-lysine 0.01% solution. Ten slides were prepared per biopsy each containing 2–4 tissue sections. Staining of the slides was carried out using the modified PAS/AB technique described by Jordan *et al.*[22] Briefly, the staining process was as follows: the slides were defrosted for 20 min; then pretreated in 100% ethanol for 10 min; after rinsing in 3% acetic acid for 2 min, the slides were stained for 2.5 h in 1% alcian blue 8G× in 3% acetic acid (pH 2.5); removed and dipped in 3% acetic acid and rinsed in running tap water for 10 min; oxidized in 1% periodic acid at room temperature for 10 min after which they were washed for 5 min; immersed for 15 min in Schiff's reagent (1 g basic fuchsin, 2 g potassium meta-bisulfite, 2 mL concentrated hydrochloric acid and 2 g activated charcoal in 200 mL distilled water); washed for 5 min in running tap water and rinsed in 0.5% sodium meta-bisulfite for 1 min per rinse, this was repeated three times, after which they were washed again in running tap water for 5 min; post-fixed in paraformaldehyde vapor at 37°C for 45 min, this was carried out by putting the slides in a desiccator containing paraformaldehyde powder; finally, the slides were mounted in water-soluble gelatine (10 g gelatine in 60 mL of distilled water mixed with 250 mg of phenol in 70 mL of glycerol).

For each biopsy 60 mucus thickness readings were taken by examining 10 different PAS/AB stained slides (one well-oriented tissue section per slide and six readings per section at intervals of approx. 0.5 cm). The measurements were performed, by one observer, using a light microscope (Leitz Wetzlar 512583, Dialux 20 EM, Germany) and specific computer software (TAS version 2.09, School of Biomedical Sciences, University of Leeds, UK). After calibrating the system with a graticule (10-μm divisions), the slide was placed on a microscope stage and examined at ×100 magnification. The appropriate section was brought into focus and the image captured with a CCD camera (model KP-M1E/K, Hitachi Denshi, Japan) and then transferred to the computer. The mucus thickness readings, in micrometers, were taken perpendicular to the gastric epithelium from the top of mucus layer to the top of the underlying epithelial cells. All mucus thickness readings were obtained without prior knowledge of the *H. pylori* status of the individual being analyzed.

Statistical analysis

Results were analyzed using SPSS software version 10.1 (SPSS, Chicago, IL, USA) and expressed as mean ± SE. Statistical significance was tested using analysis of variance (one-way and two-way ANOVA). Data transformation (log) was used when required. The level of statistical significance was taken at $P < 0.05$. Spearman correlation coefficient and multiple regression (dependent variable, gastric mucus thickness; independent variables, bacterial density, antral histology, age and sex) were used in the determination of associations between various parameters.

RESULTS

Of the 99 patients studied 69 were *H. pylori*-negative (HP–) and 30 were *H. pylori*-positive (HP+): 10 *cagA*+, 18 *cagA*–, two undetermined. All *H. pylori*-infected patients were positive on culture. The mean mucus thickness for all patients was 58.6 ± 3.9 μm. In age- and sex-matched patients there was no significant difference ($P = 0.717$) in mean mucus thickness between HP– (51.3 ± 1.1 μm, $n = 30$) and HP+ (48.8 ± 1.1 μm, $n = 30$) patients. When HP+ patients were divided into *cagA*+ and *cagA*– strains there was also no significant difference ($P = 0.784$) in mean mucus thickness between *cagA*+ (52.7 ± 1.2 μm, $n = 10$), *cagA*– (46.6 ± 1.1 μm, $n = 18$) or HP– patients (51.3 ± 1.1 μm, $n = 30$; Fig. 1). In patients without atrophy and those under 50 years old there was no significant difference in mean mucus thickness between HP– and HP+ patients or between HP–, *cagA*– and *cagA*+ patients. However, *cagA*+ patients had a mean mucus thickness that was close to those in HP– patients, but consistently greater than in *cagA*– patients, even after excluding elderly patients and those with gastric atrophy (Table 2).

In *cagA*– patients, the mean mucus thickness was significantly reduced with increased *H. pylori* colonization density (Spearman $r_s = -0.805$, $P < 0.0001$). In contrast, in *cagA*+ patients there was a weak positive, but not significant, association between mean mucus thickness and *H. pylori* colonization density (Spearman $r_s = 0.333$, $P = 0.381$; Fig. 2). Multiple regression was performed to determine whether the association between mucus thickness and *H. pylori* colonization density was influenced by factors that may affect mucus thickness, such as antral histology, patient age, and sex. In cagA–, the inverse association between mucus thick-

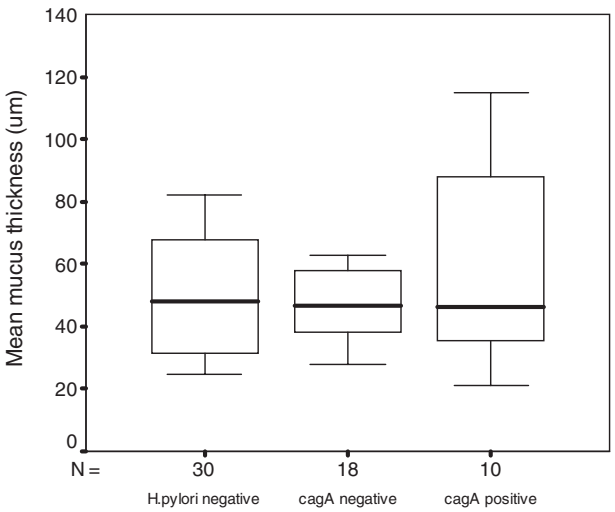

Figure 1 Box plot comparing the mean mucus thickness between age- and sex-matched *Helicobacter pylori*-negative, cytotoxin-associated gene A-negative (*cagA*–) and *cagA*+ patients. No significant difference ($P = 0.784$). *n*, no. patients.

Table 2 Mucus thickness (mean ± SE)

Group	HP– μm (*n*)	HP+ *cagA*+ μm (*n*)	HP+ *cagA*– μm (*n*)	*P*
Age- and sex-matched patients	51.3 ± 1.1 (30)	52.7 ± 1.2 (10)	46.6 ± 1.1 (18)	0.784
Matched patients ≤50 years	55.2 ± 1.2 (15)	55.7 ± 1.3 (5)	48.8 ± 1.1 (9)	0.844
Matched patients without atrophy	51.3 ± 1.1 (29)	46.7 ± 1.3 (4)	46.5 ± 1.1 (16)	0.805
Patients with atrophy	Nil	57.1 ± 1.4 (6)	46.9 ± 1 (2)	0.753

cagA, cytotoxin-associated gene A; HP+, *Helicobacter pylori* positive; HP–, *H. pylori* negative; *n*, no. patients.

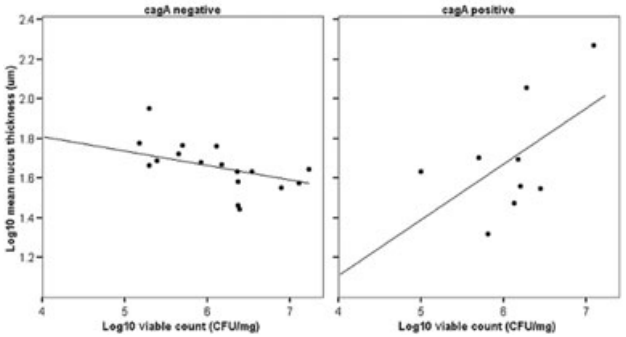

Figure 2 Correlation between mean mucus thickness in cytotoxin-associated gene A-negative (*cagA*–) and *cagA*+ infected patients and *Helicobacter pylori* colonization density. Significant ($P < 0.0001$) inverse association ($r_s = -0.805$) in cagA– patients (a), and positive but not significant association ($r_s = 0.333$, $P = 0.381$) in *cagA*+ patients (b). CFU, colony-forming units.

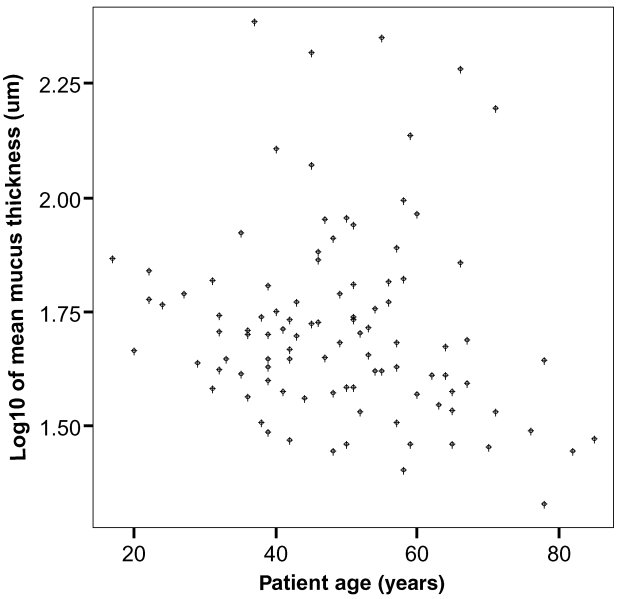

Figure 3 Correlation of gastric mucus thickness to age. Mean mucus thickness decreases with increasing age (years). Significant ($P = 0.026$) inverse correlation ($r_s = -0.223$).

ness and colonization density remained significant ($P = 0.004$), having controlled for the aforementioned factors. In *cagA*+, however, the association remained non-significant ($P = 0.359$).

In general, the older the patient the lower the mean mucus thickness because there was a significant inverse association between age and mean mucus thickness (Spearman $r_s = -0.223$, $P = 0.026$; Fig. 3). However, there was no significant correlation between age and mucus thickness within *cagA*– ($r_s = -0.323$, $P = 0.191$) or *cagA*+ patients ($r_s = -0.006$, $P = 0.987$), although there was a weak inverse relation in *cagA*– patients.

In the patients studied there were 42 men with a mean age of 46 ± 11 years and 57 women with a mean age of 51 ± 15 years. There was no significant difference ($P = 0.146$) in mean mucus thickness between male and female subjects (55.5 ± 1.1 μm and 48.2 ± 1.1 μm, respectively). In addition, within the male and female subjects there was no significant difference ($P = 0.691$ and 0.494, respectively) in mean mucus thickness between HP–, *cagA*–, and *cagA*+ patients.

Based on antral histology, five of the six patients (83%) with atrophy were infected with *H. pylori* and were all *cagA*+. The mean mucus thickness was compared in groups of patients with normal histology and those with different grades of gastritis based on antral histology alone then based on the combined (antral and body) histology. Based on antral biopsy, the mean mucus thickness in patients with mild gastritis (45.5 ± 1.1 μm, $n = 36$) and severe gastritis (33.3 ± 1.3 μm, $n = 3$) was significantly lower ($P = 0.049$ and 0.031, respectively) than those with normal histology (61 ± 1.1 μm, $n = 31$). However, the mean mucus thickness for patients with atrophy alone (78.9 ± 1.5 μm, $n = 4$), who were all *cagA*+-infected patients, was not significantly different ($P = 0.239$) from those with normal histology (Fig. 4). When both exposure factors (*H. pylori* strain and antral gastritis) were considered together (two-way ANOVA) for the effect on gastric mucus thickness, there was no significant difference in mean mucus thickness between *cagA*+, *cagA*– or HP– patients ($P = 0.625$) with different grades of gastritis ($P = 0.243$), nor was there an effect of the interaction of the two factors ($P = 0.562$). Comparison between groups based on combined histology showed no significant difference in mean mucus thickness between normal histology and the different grades of gastritis ($P = 0.289$). In addition there was no significant difference ($P = 0.140$) in mean mucus thickness in patients with normal histology ($n = 26$), active chronic gastritis ($n = 21$) and non-active chronic gastritis ($n = 36$).

DISCUSSION

The various techniques developed for mucus thickness measurement indicates the difficulty of this task, because it is not easy to preserve the viscous mucus layer in order to represent its true nature *in vivo*. Each method has been developed to overcome the problems associated with the preceding methods.

The modified PAS/AB staining technique for gastric mucus gel-layer developed by Jordan *et al.* combines the benefit of visualization of the mucus layer with staining, while at the same time avoiding tissue dehydration and shrinkage by applying modifications to the conventional PAS/AB staining technique.[22] Furthermore, their results on rat antral gastric mucus thickness (166 ± 47 μm, $n = 5$) were similar to those from adherent mucus gel thickness (after removal of the loosely adherent mucus by suction) seen *in vivo* in the anesthetized rat antral mucus (154 ± 16 μm, $n = 6$).[23] This modified technique has been shown to be superior to the conventional PAS/AB technique in preserving the mucus gel layer and has been validated for the study of mucus

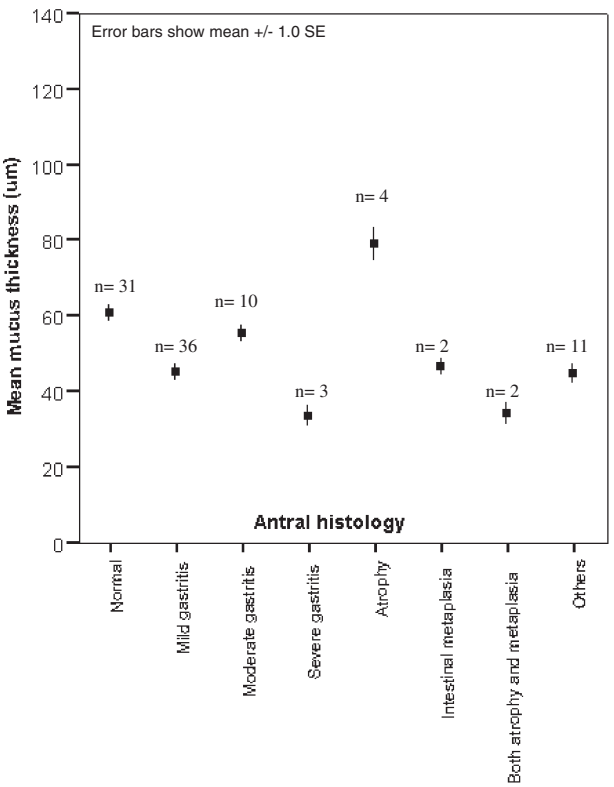

Figure 4 Mean mucus thickness of patients with normal histology compared to groups of patients with different grades of antral gastritis. Patients with mild and severe gastritis had significantly lower mucus thickness than those with normal histology. However, cytotoxin-associated gene A-positive (*cagA*+) patients with atrophy had higher non-significant mucus thickness compared with those with normal histology. Others include patients with chemical gastritis. *n*, no. patients.

thickness on human gastric biopsies. However, the drawback of this technique is the inability to exclude the small contribution to the gel thickness from the intracellular mucin stores due to reduced cytological details seen on the sections. This has been partially solved in the present study by modifying the method of mucus thickness measurements, whereby computer software linked to a light microscope was used to produce a magnified calibrated image of the tissue sections, making it easier to distinguish the top layer of the surface epithelium from the actual mucus layer. A further drawback associated with sectioning is the possibility of cutting tissue sections at oblique angles, which would influence mucus thickness readings. This was avoided in the present study by (i) properly orienting the gastric biopsy with the mucosa facing uppermost in the supporting liver tissue; (ii) labeling the aluminum foil with an arrow indicating the orientation of the biopsy; (iii) ensuring during sectioning that the microtome knife was vertical to the tissue; and (iv) discarding tissue sections that were inadvertently cut at an oblique angle as assessed by light microscopy.

Conflicting findings have been reported regarding the effect of *H. pylori* infection on the gastric mucus layer thickness, ranging from a reduction in mucus thickness[14] to no effect.[15] Sarosiek *et al.*, using unfixed mucus section technique for mucus thickness measurement on biopsy specimens, reported up to a 50% reduction in gastric mucus thickness with *H. pylori* infection.[14] Slomiany *et al.* and Sarosiek *et al.* showed that *H. pylori* produce proteases that weaken the gastric mucus layer and, therefore, decrease its viscosity, concluding that these proteases are contributing factors in the pathogenesis of gastritis and peptic ulcer disease.[27,28] However, Markesich *et al.* observed that the gastric mucus viscosity was significantly higher in patients infected with *H. pylori*.[29] Consequently, they argued against the hypothesis that degradation of the gastric mucus by *H. pylori* is important in the pathogenesis of peptic ulcer disease. Later studies, however, have failed to show the production of proteases by *H. pylori*.[30,31] Newton *et al.*, in their study on mucus thickness using a new technique of staining by PAS/AB, have reported no significant difference in mucus thickness between *H. pylori*-positive and *H. pylori* negative-patients, but *H. pylori* infection does reduce mucin polymeric structure.[15] This latter study showed that *H. pylori* infection alone does not reduce the thickness of the adherent mucus barrier and thus a protective stable unstirred surface gel layer is preserved. It is only with *H. pylori*-associated gastric atrophy or advancing patient age that there is a significant reduction in mucus thickness.[15,32]

In the present study, no significant difference in mean mucus thickness in age- and sex-matched patients between *H. pylori*-infected ($n = 30$) and -uninfected ($n = 30$) patients was observed. This supports the earlier findings of Newton *et al.* on a smaller number of patients (20 HP+ and 20 HP–), but they did not distinguish between those infected with *cagA*+ and *cagA*– *H. pylori*.[15] Whereas in *cagA*– patients mucus thickness significantly decreases in the presence of high bacterial colonization density, in *cagA*+ patients there was no relation between increased mucus thickness and a higher bacterial colonization, although a weak positive association was apparent. One could argue that the association between *H. pylori* quantification by culture and mucus gel thickness could be because *H. pylori* may be easier to grow if the mucus layer is thin.

Newton *et al.* demonstrated significant thinning of the adherent gastric antral mucus layer thickness in HP+ individuals with increased age.[32] In the present study, a significant correlation between increased age and thinning of the gastric mucus thickness was observed. However, there was no correlation between mean mucus thickness and age in *cagA*+ and *cagA*– patients, although a weak, but non-significant relationship was present between increased age and thinning of the mucus layer in *cagA*– patients. The findings of decreased thickness with increased age by Newton *et al.* in the presence of HP+ infection[32] could be explained if most of their patients were *cagA*–.

Although age, sex and antral histology did not influence gastric mucus thickness in the presence of *H. pylori* infection, it is important to note the non-significant trend of increased mucus thickness in (i) male *cagA*+-infected patients compared to female patients; (ii) *cagA*+ patients with gastric atrophy; and (iii) cagA+ patients compared to *cagA*– patients. These observa-

tions suggest that there is a tendency for increased mucus thickness in the presence of *cagA*+ infection, which may then play a role in protecting *cagA*+ strains by the mucus layer. This would then lead to their persistent inflammatory effect that could account for the high association of *cagA*+ infection with severe gastric diseases,[6–8] especially the risk of gastric cancer.[10] However, this needs to be examined by larger studies in geographic areas with high *H. pylori* prevalence because the differences observed could be due to type II error. Alternative explanations for altered mucus gel thickness in the presence of *H. pylori* infection could be increased mucus secretion or synthesis, either of which could be driven directly by *H. pylori* or indirectly by inflammation.

In vivo mucus thickness measurement is the best method to assess thickness without any deterioration of the mucus layer associated with its preparation for the *in vitro* measurements. The method of *in vivo* gastric mucus layer thickness measurements in the anesthetized rats by Atuma *et al.*[23] would be ideal, but it cannot be applied in humans. Future techniques assisted by endoscopy to measure human mucus thickness *in vivo* are a desirable future development.

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