Published in final edited form as:
Helicobacter. 2010 August ; 15(4): 251–258. doi:10.1111/j.1523-5378.2010.00765.x.

Role of mucin Lewis status in resistance to *Helicobacter pylori* infection in pediatric patients

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Abstract

Background—*Helicobacter pylori* causes gastritis, peptic ulcer and is a risk factor for adenocarcinoma and lymphoma of the stomach. Gastric mucins, carrying highly diverse carbohydrate structures, present functional binding sites for *H. pylori* and may play a role in pathogenesis. However, little information is available regarding gastric mucin in children with and without stomach diseases.

Material & Methods—Expression of mucins and glycosylation was studied by immunohistochemistry on gastric biopsies from 51 children with and without *H. pylori* infection and/or peptic ulcer disease.

Results—In all children, MUC5AC was present in the surface epithelium and MUC6 in the glands. No MUC6 in the surface epithelium or MUC2 was detected in any section. The Le^b and Le^a blood-group antigens were present in the surface epithelium of 80% and 29% of children respectively. *H. pylori* load was higher in Le^b negative children than in Le^b positive individuals (means ± SEM 17.8±3.5 vs 10.8±1.5; $p < 0.05$), but there was no correlation between Le^a or Le^b status and gastritis, nodularity, and gastric or duodenal ulcer. Expression of sialyl-Le^x was associated with *H. pylori* infection, and DU.

Conclusions—Mucin expression and glycosylation is similar in children and adults. However, in contrast to adults, pediatric *H. pylori* infection is not accompanied by aberrant expression of MUC6 or MUC2. Furthermore, the lower *H. pylori* density in Le^b positive children indicates that *H. pylori* is suppressed in the presence of gastric mucins decorated with Le^b, the binding site of the *H. pylori* BabA adhesin.

Keywords

Gastric Mucosa; Mucins; *Helicobacter pylori*; Carbohydrate Antigens; Pediatric; gastritis; peptic ulcer

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Introduction

H. pylori is the main causative agent of gastric and duodenal ulcers in adults and children (1, 2). However, only a fraction of *H. pylori* infected subjects develop ulcers, an observation attributed to concurrent differences in bacterial virulence as well as host and environmental factors (3). One virulence factor potentially involved in the pathogenesis of peptic ulcer disease (PUD) is the blood antigen binding adhesin BabA (4–7). The majority of *H. pylori* strains investigated carry the *babA* gene, (4, 6), and since the BabA adhesin adheres to gastric blood group antigens expressed on epithelial cells and mucus (8, 9), the expression of specific host receptors to bacterial adhesins are likely to play a role in the outcome of the infection (10).

Gastric mucosal surfaces are covered with a mucus layer primarily composed of secreted mucins. This mucus layer is continuously secreted and transports away trapped material. The mucus polymer is formed by oligomeric mucin glycoproteins that provide a matrix for a rich array of anti-microbial molecules. These mucins inhibit bacterial access to the mucosa by binding microbes (9) and, in the case of MUC6 oligosaccharides, by providing a direct antimicrobial activity (11). In adults, gastric MUC5AC and MUC6 are the major secreted mucins and are produced by the surface epithelium and by the glands, respectively (12). In the fetal stomach, in addition, MUC2 and MUC5B are also expressed (13) and mucin gene expression can be “fetal-like” in adults with gastric precancerous lesions and cancer (14, 15). Other disease-related changes in mucin expression are the presence of MUC2 and MUC6 in intestinal metaplasia and in the surface epithelium of *H. pylori* infected adults, respectively (16).

Each mucin carry a vast array of oligosaccharide structures, and the glycosyltransferases expressed by the individual determines the type of carbohydrate structures present on the secreted mucins. The H1 structure is made by the secretor gene and the majority of people carry this structure and are thus referred to as ‘secretors’ (17). Individuals may also express the Lewis gene (90% of the Caucasian population) and express the Le^b histo-blood group antigen if they are secretors and Le^a if they are non-secretors (Table 1)(17, 18). A third secretor phenotype, the weak-secretor phenotype Se^w, is characterized by expression of both Le^a and Le^b antigens (19). In the healthy adult stomach, the surface epithelium expresses Le^a and Le^b structures and the Le^b structure is expressed on the MUC5AC mucin (9, 12, 20).

H. pylori is mainly found within the mucus layer and it is also attached to, or within, gastric epithelial cells.(21, 22) Mucins have a high and specific binding capacity for *H. pylori* (9, 23, 24). As a result, mucins function as decoys for bacterial binding (23) and they prevent most *H. pylori* from approaching epithelial cell surface. In the human-like rhesus monkey model (25), animals secreting mucins with higher *H. pylori* binding capacity developed lower *H. pylori* density infection and less gastritis (10, 25). Similarly, humans with primary Sjögren’s syndrome produce smaller quantities of mucins and have more *H. pylori*-associated pathology (26), further suggesting that the ability of secreted mucins to bind *H. pylori* protects the gastric epithelium. Three different modes of adhesion have been implicated in *H. pylori* binding to gastric mucins: the Le^b and sialyl-Le^x/sialyl-Le^a binding adhesins and a charge/low pH mode of adhesin (9, 23, 24, 27, 28). In the healthy stomach, *H. pylori* binding to the gastric MUC5AC via the Le^b mucin structure is the dominating mode of adhesion (9, 23, 24, 27, 28). Mucin glycosylation is predominantly neutral in the healthy stomach, whereas both sulfated and sialylated structures appear in infection, inflammation and cancer (20, 28). These disease-related changes in mucin expression and glycosylation modify the *H. pylori* adhesion targets,(10) and enhance *H. pylori* binding ability of secreted mucins (24).

Gastric colonization with *H. pylori* usually begins in childhood (29), although, in contrast to adults, gastro-duodenal ulcers and intestinal metaplasia are rare in children (30). Furthermore, children with *H. pylori* infection predominantly have pangastritis instead of the diffuse antral or multifocal atrophic gastritis described in adults (31). In addition, the numbers of inflammatory cells present in *H. pylori*-infected biopsy specimens is higher in children than adults (30) although IFN-γ secretion in the stomach of *H. pylori*-infected patients is lower in children than in adults (32). Finally, *H. pylori* contact with microvilli is higher in children compared to adults whereas the more intimate adhesion with abutting or adhesive pedestals dominate in adults (33).

Given these differences and the fact that the effect of *H. pylori* infection on mucin expression and glycosylation was extensively studied in adults (9, 15, 34–44) but not in children, it is important to investigate mucin expression and glycosylation in pediatric *H. pylori* infection. Here, we determined the expression and morphological location of the MUC5AC, MUC6, MUC2, MUC5B mucins and the Le^a, Le^b, sialyl-Le^a, and sialyl-Le^x carbohydrate structures (Table 1) in children and determined whether these parameters were different in the presence of gastritis, peptic ulcer disease and/or *H. pylori* infection.

Materials and Methods

Materials

Monoclonal antibody against the MUC5AC mucin (45M1) and Iodoacetamide were obtained from Sigma, BSA from Serva and 1,4-dithiothreitol (DTT) from Merck. Polyclonal antibodies against MUC6 (LUM6-3),(45) MUC5B (LUM5B-2 (46)) and MUC2 (LUM2-3 (47)) were kind gifts from Professor Ingemar Carlstedt, Lund University, Lund, Sweden. Monoclonal anti-Le^b antibody (clone 2–25 LE) and anti Le^a (7LE) were kind gifts from Dr. J. Bara, INSERM, France. Antibodies against Le^a (BG-5), Le^b (BG-6), were from Signet pathology systems, antibodies against sialyl-Le^x (clone KM93) and sialyl-Le^a (clone 1H4) from Seikagaku America, Biotinylated goat anti mouse and goat anti rabbit antisera, Strept AB complex/HRP from Vector and biotin blocking system from Dako. Thiopropyl Sepharose 6B was from Amersham Bioscience. DAB (3,3-diaminobenzidine tetrahydrochloride) was from (Sigma).

Study Population

Specimens used herein were harvested in a study approved by the Institutional Review Boards of Walter Reed Army Medical Center and the Uniformed Services University of the Health Sciences. The study included 51 children with either normal endoscopy, nodularity, gastric ulcer or duodenal ulcer as recently reported (48). Mean age was 12.2 years (range: 3 to 18; Since the youngest patients are 3 years old, mucin intake via mothers milk is not likely to confound the results from this study), and the ethnic distribution was 53% Caucasians, 27% African-Americans, 14% Hispanics, and 6% Asians. The indications for endoscopy included abdominal pain (n=38, 74%), nausea and vomiting (n=33, 65%), upper gastrointestinal bleeding (n=8, 16%), weight loss (n=4, 8%) and heartburn (n=8, 16%). The age, gender, and *H. pylori* positivity are listed in table 2. The ethnicity and subjective symptoms of the patients were not significantly different among the endoscopic groups, as reported (48).

Endoscopy

After obtaining informed consent, all patients underwent upper gastrointestinal endoscopy with the XP-20, P140, or GIF-100 endoscope (Olympus). The endoscopy reports were graded as follows: normal (n = 14), nodularity (n = 18), gastric ulceration (GU, n = 8) and duodenal ulceration (DU, n = 11).

Histology

Serial paraffin sections from archived biopsies (4 μm) were stained with either H&E, according to Genta (49), or for *in situ* hybridization (48) and imunohistochemistry (see below). Chronic and acute inflammation, intestinal metaplasia, atrophy, and *H. pylori* density were scored according to the Updated Sydney System (50). Inflammation and atrophy scores represent the degree of either acute or chronic inflammation (0=normal, 1=mild, 2=moderate, and 3=severe). A pediatric pathologist blinded to the results of the endoscopy, Genta stain, *in situ* hybridization and immunohistochemistry performed the histological assessment (48).

H. pylori quantification

Fluorescence *in situ* hybridization (FISH) was used to determine concurrently the expression of *H. pylori 16S rRNA* and *cagA* using specific probes as described (48). Briefly, the *16S rRNA and cagA* probes were labeled with biotin and digoxigenin, respectively, and binding was detected with Avidin-Texas red and anti-digoxigenin-FITC, respectively. Specific controls of method were also performed to verify specificity of the technique. Specimens were considered *H. pylori* positive when Genta-stained organisms of typical curved-shape morphology were present and if there was a positive FISH reaction for *H. pylori 16S rRNA*. An Eclipse E-800 Nikon microscope was used to examine the sections. Morphometric quantification of *H. pylori* by Genta and expression of *16S rRNA* and *cagA* was performed using a previously published point-counting stereological technique (22). This method utilizes an intraocular reticule (No. Kr409, Klarman Rulings, Inc. Litchfield, NH) that has a 27 mm diameter grid covering 3,577 μm^2, thus 14,314 μm^3 for 4 μm-thick sections. Bacterial clusters that were Genta-stained (and displayed the typical curved shape) or expressed *H. pylori 16S rRNA* were quantified at 400X.

Immunohistochemistry

MUC2, MUC5AC, MUC5B, MUC6, Le^a, Le^b, sialyl-Le^a and sialyl-Le^x immunohistochemistry was performed on formalin fixed, paraffin-embedded tissue sections according to standard procedures as previously described (25). The investigator scoring sections stained via immunohistochemistry (SL) was unaware of the clinical diagnosis or *H. pylori* infection density when performing the analysis. Human adult gastric and intestinal biopsies were used as positive controls.

Purification of anti-MUC2 antibodies by affinity chromatography of LUM2-3 antiserum

The synthetic peptide (NGLQPVRVEDPDGC) used for production of the LUM2-3 antiserum (47) was covalently conjugated to Thiopropyl Sepharose 6B (2 mg peptide/g dry Thiopropyl Sepharose 6B) according to the protocol supplied by the manufacturer. The LUM2-3 antiserum was diluted 1:10 in 10 mM Tris buffer, pH 7.5 and applied to the column at a flow rate of 0.05 ml/min. The column was washed with 10 mM Tris buffer, pH 7.5 and 0.5 M NaCl in 10 mM Tris buffer, pH 7.5 before bound antibodies were eluted with 0.1 M Triethylamine pH 11.5.

Statistics

Data are reported as frequency (percent) or means ± standard error. Student's t test for independent samples was used to compare means between groups, and the chi square test was used to compare proportions. A $p < 0.05$ was considered statistically significant.

Results

H. pylori infection

H. pylori were observed either free in the lumen or adherent to the foveolar and epithelial surface (Figure 1). As previously reported (48), Genta staining and FISH demonstrated their presence in half of the children with normal endoscopy, and in all or almost all patients with nodularity, DU or GU. In addition, FISH showed that *H. pylori* and *cagA* expression was significantly higher in ulcer patients compared to children with normal endoscopy (48).

Mucin expression and tissue localization

MUC5AC was detected in the surface/foveolar epithelial cells in all sections. The cytoplasm of the epithelial cells was mainly comprised of MUC5AC positive mucus granules, and in sections where the mucus layer had been retained, the mucus layer was also positive for MUC5AC (Figure 2A). MUC6 was located in the antral glands and in the larger oval neck cells of the corpus (Figure 2B). Secreted MUC6 was detected in the lumen of the foveolae, but no MUC6 was detected in the surface/foveolar epithelium in any of the biopsies.

Sialomucins were mainly detected in the neck region. Neither MUC2 nor MUC5B were detected in any of the biopsies. There were fewer mucin producing cells in the epithelia with intense inflammation and thicker lamina propria. As a result, the tall normal epithelial cells loaded with mucus were replaced by short or flat cells containing little mucus in the mucin thecae. However, no difference in tissue localization of mucins was detected among children with and without gastritis, PUD and/or *H. pylori* infection.

Lewis antigens

In the surface/foveolar epithelium, Le^b (Figure 2C) and Le^a were detected in 80% and 29% of children (Table 3), respectively, but not in the glands of any of the children. In biopsies where Le^b and/or Le^a was present, the mucous cells of the entire surface and foveolar epithelium tended to be positive, although there were slight variations in stain intensity between individuals. Le^b and Le^a thus have tissue localizations similar to the MUC5AC mucin (Compare Figure 2A with 2C). The Le^{b+}/Le^{a-} phenotype was inversely associated with presence of sialomucin (13/29 [45%] children with this phenotype had sialomucin present, compared with 15/20 [75%] of children with other phenotypes, p =0.038).

Sialylated Lewis antigens

Sialyl-Le^a was present in mucus producing cells, secreted mucin and lamina propria in 60% of the children and sialyl-Le^x was positive in 29% of the children (Figure 2D and E). In the 14 sialyl-Le^x positive individuals, secreted mucins and/or mucin producing cells were sialyl-Le^x positive in 11, whereas only the lamina propria was positive in the other three. Only a minority of the cells stained for sialyl-Le^x, and the staining occurred most often in the antral foveolar epithelium (Figure 2D). Expression of sialyl-Le^x was associated with the Le^{a+} phenotype (9/13 (69%) vs. 7/34 (21%), p = 0.002).

Lewis expression, level of infection, and endoscopic diagnosis

H. pylori density was higher in Le^b-negative children than in Le^b-positive individuals (means ± SEM: 17.8±3.5, n=10 versus 10.8±1.5, n=39; p =0.045). In addition, presence of sialyl-Le^x was associated with *H. pylori* infection and DU; 42% (16/38) of *H. pylori* infected patients were sialyl-Le^x positive and 67% (6/9, p= 0.023) of DU patients were sialyl-Le^x positive compared to 0% (0/9 p = 0.018) without *H. pylori* infection and DU. However, no significant relation was found between Le^b status and endoscopical diagnosis (normal, nodularity, GU or DU).

Discussion

Here, we observed that the histological location of the MUC5AC and MUC6 mucins as well as Lewis fucosylation and sialylation are similar in normal pediatric and adult gastric tissue but that, in contrast to adults (16), pediatric *H. pylori* infection was not accompanied by aberrant expression of MUC6 or MUC2. In another study, MUC2 was detected in a few foveolar and surface cells in *H. pylori*–infected children, but the observation was made in only 2/18 children.(51). No aberrant expression of MUC6 in the surface or foveolar epithelium was found in the present study, although MUC6 has been shown to be aberrantly expressed in the surface epithelium in 72% of *H. pylori*-infected adults (16). These differences may be due to the fact that abnormal expression of MUC2 and MUC6 require a longer *H. pylori* exposure than that experienced by our group of children. This hypothesis is supported by the observation that Rhesus monkeys followed for 10 months after *H. pylori* inoculation did not show changes in MUC5AC, MUC6 or MUC2 tissue localization (10).

In children, as in adults, the cytotoxin-associated A (*cagA*) gene is the most frequently implicated virulence factor associated with increased risk of PUD (1). We recently demonstrated that the fraction of *H. pylori* bacteria expressing *cagA in situ* was higher in children with peptic ulcer (48). However, no association between host Lewis antigen expression and *H. pylori cagA* expression was detected in this study.

In the present group of children, prevalence of Le^a and Le^b histo-blood group antigens expression in the foveolar epithelium was similar to that found in adults (52). No significant correlation was found between Le^a or Le^b status and endoscopic findings of gastritis, nodularity, gastric ulcer, duodenal ulcer, or *H. pylori* infection. This is in agreement with previously published data from adults showing that the Le^b host phenotype is not associated with *H. pylori* infection or PUD (38). However, we found that *H. pylori* density was significantly higher in Le^b negative individuals than in Le^b positive individuals, which is similar to results obtained in Rhesus monkeys (10). Secreted mucins function as decoys for bacterial binding to the epithelial surface, and they are produced in large amounts and constantly wash the mucosal surfaces (23). Mucins efficiently bind *H. pylori* via Le^b, reducing the number of *H. pylori* available to bind to the mucosal cell surfaces (9, 10, 24). In the human-like rhesus monkey model, we previously observed that the Le^b positive mucins act as functional glyco-decoys and clearance factors and therefore reduce infection density. Taken together, these results demonstrate that the secretor and Lewis status play an intrinsic role in resistance to *H. pylori* infection and suggest that the fucosylated secretor antigens constitute interactive members of the young human mucosal innate immune system. In contrast to the results from the young humans and rhesus monkeys, Taiwanese adult patients expressing Le^b have a higher *H. pylori* density than those who do not, and *H. pylori* density increases with Le^b expression (42). This difference may be due to the fact that the protective function of Le^b may only be functional in young individuals because the adherent gastric antral and duodenal mucus gel layer thins with advancing age and duration of *H. pylori* infection (53).

In conclusion, the expression and tissue localization of mucins and Le^a, Le^b, Le^x, sialyl-Le^x and sialyl-Le^a were similar in uninfected children and adults. However, in contrast to reports in adults, *H. pylori* infection was not commonly accompanied by aberrant expression of MUC6 or MUC2 in children, which may relate to duration of exposure to the bacterium. Furthermore, in contrast to reports in adults, Le^b negative children had a greater *H. pylori* density than Le^b positive children. These results strongly indicate that the secretor and Lewis status plays an intrinsic role in resistance to *H. pylori* infection and suggest that the fucosylated secretor antigens constitute interactive members of the young human mucosal innate immune system.

Acknowledgments

We thank Cara Olsen (Uniformed Services of the Health Sciences, Bethesda, Maryland, USA) for critical review of statistical analysis. The work was supported in part by grants from The Swedish Research Council (SL) and the National Institutes of Health (AD) (CA82312).

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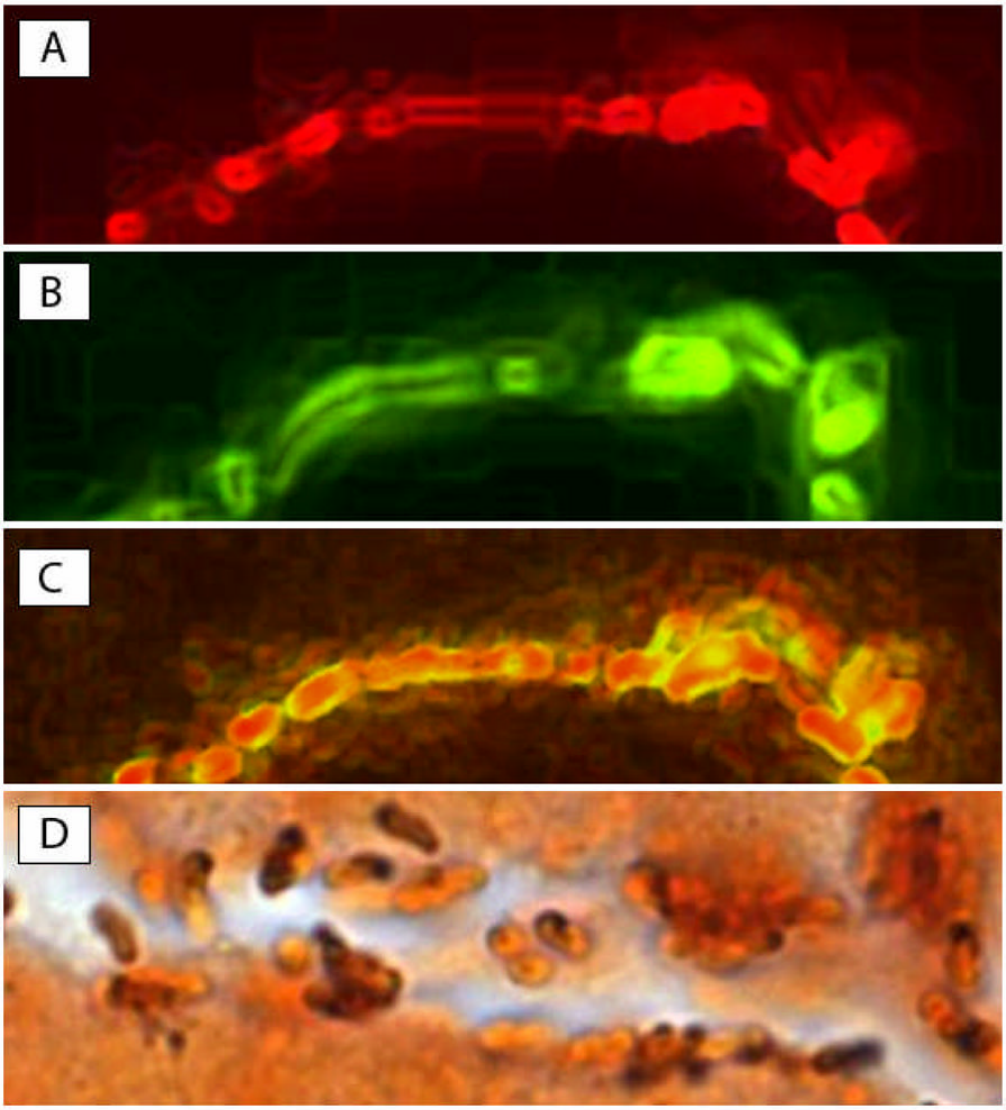

Figure 1. *H. pylori* in antral gastric gland

H. pylori colonization in superficial gland of a child with duodenal ulcer in two 5-μm-thick serial sections (Original magnification X1000). (A) 1*6S rRNA* in red (FISH-Texas red); (B) *cagA* in green (FISH-FITC); (C) merge of A and B, demonstrating colocalization of *16S rRNA* and *cagA* (in yellow) in the same bacteria. Note that only cagA (green) is expressed in the outer part of bacteria while both *16S rRNA* and *cagA* are co-localized in the center (yellow); (D) Genta stain of a gastric gland, showing many curved *H. pylori*-shaped bacteria colonizing the lumen mucus or adherent to the apical part of mucus-secreting cells. Note that some of the bacteria appear intensely stained black by silver, while others are weakly stained (in part or completely). These variations may represent different capture of silver stain and may be due to the presence of mucus on part of the bacterial membrane.

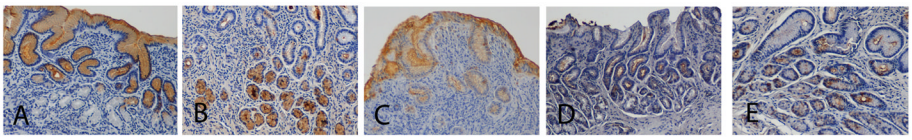

Figure 2. Tissue localization of mucins and carbohydrate antigens

Section from a *H. pylori* negative patient was stained brown using immunohistochemistry for MUC5AC (A). Sections from *H. pylori* positive patients were stained brown for MUC6 (B), Le^b (C), Sialyl-Le^x (D) and Sialyl-Le^a (E).

Table 1

Structure of Le^a, Le^b, sialyl-Le^a and sialyl-Le^x

Lewis antigen	Structure
Le^a	Galβ1-3GlcNAc | 1,4 Fucα
Le^b	Galβ1-3GlcNAc | | 1,2Fucα 1,4Fucα
Sialyl-Le^a	NeuAcα2-3Galβ1-3GlcNAc | 1,4Fucα
Sialyl-Le^x	NeuAcα2-3Galβ1-4GlcNAc | 1,3Fucα

Table 2

Demographics and *H. pylori* prevalence in all patients.

	Normal (n=14)	**Nodularity (n=18)**	**GU (n=8)**	**DU (n=11)**
Mean Age [years (range)]	13.1 (10–17)	12 (4–17)	10.4 (3–17)	12.7 (8.5–17)
Males [N (%)]	7 (50)	10 (53)	5 (63)	9 (82)
***H. pylori* positive [**N (%)]	7 (50)	18 (100)	6(75)	10(91)

Table 3

Individuals with clinical diagnosis of the different Lewis phenotypes.

Lewis phenotype	All individuals n=49*	Controls n=6	*H. pylori* infection n=39	Gastritis n=39	Nodularity N=18	Gastric Ulcer n=8	Duodenal ulcer n=10
Le^{b+}	39 (80%)	5 (83%)	31 (79%)	31 (79%)	13 (72%)	6 (75%)	9 (90%)
Le^{a+}	14 (31%)	2 (33%)	11 (29%)	11 (29%)	3 (17%)	3 (38%)	4 (40%)
Le^{b+},Le^{a+}	11 (22%)	2 (33%)	9 (23%)	9 (23%)	2 (11%)	1 (13%)	4 (40%)
Le^{b+},Le^{a-}	28 (59%)	3 (50%)	21 (54%)	20 (51%)	11 (61%)	5 (63%)	5 (50%)
Le^{a+}, Le^{b-}	3 (6%)	0	3 (8%)	3 (8%)	1 (6%)	2 (25%)	0
Le^{a-}, Le^{b-}	7 (14%)	1 (17%)	6 (15%)	7 (18%)	4 (22%)	0	1 (10%)

Insufficient material was available to analyze Lewis type in 2 of the 51 patients. Some patients had several clinical diagnoses, which is why the numbers of gastritis, nodularity, GU and DU add up to more than the number of patients in the study.