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ORIGINAL ARTICLE

Membrane-Bound Mucins and Mucin Terminal Glycans Expression in Idiopathic or *Helicobacter pylori*, NSAID Associated Peptic Ulcers

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Abstract
Background The ratio of *Helicobacter pylori*/NSAID-negative gastric ulcers is increasing. Idiopathic gastric ulcers have unique clinical and endoscopic features, and are associated with more bleeding complications and a higher mortality. Alterations in gastric mucin expression and sialylation pattern may be important in ulcer pathogenesis.
Aims The purpose of this study was to determine the expression pattern of membrane-bound mucins and side chain sugars in *H. pylori* associated-, NSAID-, and idiopathic-gastric ulcers.
Methods We randomly selected 92 patients with *H. pylori* (group 1, $n = 30$), NSAID (group 2, $n = 18$), combined *H. pylori* and NSAID associated gastric ulcers (group 3, $n = 24$), and patients with idiopathic gastric ulcers (group 4, $n = 20$). Immunohistochemistry for T-cell CD4/CD8, MUC1, MUC4, MUC17, and ECA and SNA lectins staining was performed on sections from the ulcer margins. Inflammation score was assessed according to the Sydney system.
Results Bleeding and mortality rates were significantly higher in group 4. CD4 positive T cell count was higher in *H. pylori* positive patients ($P = 0.009$). Staining intensity of MUC17 was higher in group 1 than in group 4, foveola and glands alike, with 11.50 ± 3.47 versus 6.80 ± 4.02, and 9.61 ± 4.26 versus 7.59 ± 3.26, respectively ($P < 0.0001$). This was a mirror image with MUC1. SNA lectin staining was increased in group 4, in parallel to MUC1 expression, indicating more abundant α2-6 sialylation in that group.
Conclusions Cytoplasmic MUC17 staining was significantly decreased in the cases with idiopathic ulcer. The opposite was demonstrated for MUC1. This observation might be important, since different mucins with altered sialylation patterns likely differ in their protection efficiency against acid and pepsin.

Keywords Idiopathic ulcer · Mucin · MUC1 · MUC4 · MUC17 · SNA · ECA · *Helicobacter pylori*

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What Is Already Known About This Subject?

- The fraction of gastric ulcer not related to *H. pylori* infection or NSAID therapy is increasing.

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- Idiopathic gastric ulcers have unique clinical and endoscopic features, and are associated with more bleeding complications and a higher mortality.
- Alterations in gastric mucin expression and sialylation pattern may be important in ulcer pathogenesis.

What Are the New Findings?

- Cytoplasmic MUC17 staining was significantly decreased in the cases with idiopathic ulcer.
- The opposite was demonstrated for MUC1.
- Staining for sialic acid mucin residue was significantly increased in the cases with idiopathic ulcer.

How Might It Impact on Clinical Practice in the Foreseeable Future?

- This observation might be important, since different mucins with altered sialylation patterns likely differ in their protection efficiency against acid and pepsin.

Introduction

Helicobacter pylori (*H. pylori*) infection and non-steroidal anti-inflammatory drugs (NSAIDs) are the leading causes of gastric ulcer [1–5]. However, in up to 39 % of cases neither of these risk factors are identified [6]. While it may be prudent to exclude rarer causes of gastric ulcer such as malignancy, Zollinger-Ellison syndrome or systemic mastocytosis, in most of the cases *H. pylori*/NSAID-negative ulcers are apparently idiopathic.

Mucins are high-molecular-weight glycoproteins, which are heavily glycosilated with O-linked oligo-saccharides and *N*-glycan chains, linked to a protein backbone. There are at least 21 mucin (MUC) genes known in the human genome. These genes encode two groups of mucins: secreted mucins and membrane-bound mucins. The main mucins expressed in the stomach are MUC1 (membrane-bound) and MUC5AC and MUC6 (secreted mucins). It has been proposed that defects in gastric mucin quality or quantity play a role in the pathogenesis of *H. pylori*/NSAID-negative ulcers [7]. MUC5AC is secreted by surface foveolar cells and forms the bulk of the adherent unstirred mucous layer, whereas MUC6 is secreted by neck and gland cells. These two mucin proteins remain segregated within the mucous gel in a laminated linear arrangement [8]. NSAIDs disrupt the production of prostaglandine-E2 (PGE2) which mediates mucin secretion. *H. pylori* similarly decreases mucin synthesis via inhibition of galactosyltransferase [9], fighting mucin's inherent glycan-related antibacterial properties [10]. In a previous study we found no difference in the secreted mucins MUC5AC and MUC6 expression in gastric ulcers associated with *H. pylori* infection or NSAID therapy [11].

The pattern of membrane-bound mucins and sialic acid expression in idiopathic peptic ulcer disease has never been examined. As representatives of gastric membrane-bound mucins we choose MUC1, MUC4 and MUC17 since they were described to be part of the normal glycocalyx and mucin barrier of the stomach. MUC17 is also considered a protective gene against cancer and inflammation [12]. *Sambucus Nigra Agglutinin* (SNA) is used as a probe to detect sialic acid (Sias) in α2-6 glycosidic linkage to underlying glycans, most commonly galactose [13], and *Erythrina Cristagalli* Agglutinin (ECA) is used as a probe to detect *N*-acetyllactosamine (Galβ1-4GlcNAc) [14], which is the most common glycan structure underlying sialic acids in α2-6 linkage. *N*-acetyllactosamine is exposed when the sialic acid is removed. Both lectins were chosen since sialic acid residues on mucin carbohydrate side chains are considered important for the mucin protective effect.

The aim of the present study was to identify the clinical and endoscopic features, and gastric membrane-bound mucins expression pattern in peptic ulcer disease, positive and negative for *H. pylori* infection or NSAID therapy. We also looked for sugar (glycan) side chains peripheral composition by lectin staining, intraepithelial T-cell lymphocyte distribution and inflammation score.

Methods

Patients

Approval was granted by the ethics committee of Rabin Medical Center. Non-consecutive patients who underwent routine or emergency upper endoscopy at the Department of Gastroenterology, Rabin Medical Center, Beilinson Hospital, and were assigned an endoscopic diagnosis of gastric ulcer of 5 mm in size or larger, between 2003 and 2005, were identified using an established computerized endoscopy reporting system. We chose only patients with gastric ulcers and not peptic duodenal ulcers, since in cases of gastric ulcer one always takes biopsies to exclude gastric cancer. This is not the case in peptic duodenal ulcers. Clinical parameters were recorded, including patient age and gender, major indication for upper endoscopy, concomitant diseases and the use of aspirin and NSAIDs in the previous 3 months. Patients on antibiotics, proton pump inhibitor and bismuth treatment, or those with upper gastrointestinal bleeding were excluded. Endoscopic

parameters were recorded including ulcer site, size and number. *H. pylori* status was determined via histological detection on biopsies taken from the ulcer margins, gastric body or antrum and/or the rapid urease test and/or ^{13}C-urea breath test performed within 3 months. For positivity, one of these tests was acceptable, and for negativity at least two. Exclusion criteria included cases where no biopsies were taken from the ulcer margin, and where biopsies revealed neoplasia. Patients with clinical or histological evidence of Zollinger Ellison syndrome, gastrointestinal malignancy, eosinophilic gastroenteritis, systemic mastocytosis, and patients receiving biphosphonates, potassium salts or iron, were also excluded.

Tissue Samples and Mucins Immunohistochemistry

Formalin fixed, paraffin embedded tissue from ulcer margins were obtained from the Pathology Department. Where additional biopsies of gastric body or antrum were taken at endoscopy, these too were obtained. Paraffin embedded blocks were cut into 4-μm thick sections. Slides were deparaffinized in xylene and rehydrated using a graded ethanol series. Antigen was retrieved by boiling the slides in a microwave oven for 15 min in 0.01 mol/L citrate buffer (pH 6.0). Endogenous peroxydase was blocked with a 3 % H_2O_2-methanol solution, and the slides were incubated in 10 % normal goat serum for 30 min to prevent nonspecific staining. The tissue sections were then incubated overnight at 4 °C with primary antibody (anti-MUC1, anti-MUC4 or anti-MUC17, 1:100; Santa Cruz, CA). The standard biotin–streptavidin–peroxidase method was then used, and the sections were lightly counterstained with hematoxylin. Histologically normal gastric biopsies were used as positive controls for MUC1, MUC 4 and MUC17. The sections incubated with phosphate-buffered saline (0.01 mol/L, pH 7.4) instead of primary antibody were used as negative controls.

SNA and ECA Lectin Fluorescent Staining

The tissues were blocked with 1 % BSA/PBS (bovine serum albumin, Sigma-Aldrich) for 10 min, and incubated with fluorescein-conjugated *Sambucus nigra* agglutinin (SNA-FITC, 1:1,000 dilution, Vector Labs) in HEPES/NaCl buffer (10 mM HEPES, 150 mM NaCl pH 7.5) for 1 h at room temperature [13, 14]. The nuclei were counterstained with DAPI, and tissues were mounted with aqueous mounting medium (Vector Labs).

Immunohistochemistry Lectin Staining

Endogenous peroxidase was blocked with H_2O_2, and endogenous biotin was blocked with Avidin–Biotin blocking kit (Vector labs) according to manufacturer's instructions. Tissues were further blocked with 1 % BSA/TBST (0.05 M Tris HCl, 150 mM NaCl pH 8.0, 0.1 % Tween 20) for 10 min, and incubated for 30 min with biotinylated-SNA (1:1,000 dilution, Vector Labs) or with biotinylated-ECA (1:2,500 dilution, Vector labs) in 1 % BSA/TBST supplemented with 10 mM $CaCl_2$ and 10 mM $MnCl_2$ at room temperature. Tissues were washed and incubated for 30 min with horseradish peroxidase conjugated streptavidin (Streptavidin-HRP, 1:500 dilution, Jackson Immunoresearch), followed by 5 min incubation with Vector Blue substrate (Vector Labs) in 0.1 M Tris/levamisole. Nuclei were counterstained with nuclear fast red for 30 min, and tissues were mounted with aqueous mounting medium (Vector Labs).

Staining Interpretation

All slides were scanned by the NanoZoomer and digitalized (NanoZoomer 2.0 series, Hamamatsu, Japan). Whole slide scans allow complete review, examination and analysis of all parts of the tissue, accurately assigned identical low or high power microscopy fields. Cytoplasm staining was assessed in at least ten high-power fields by two observers at two sites: the foveola and the glands. The range of cytoplasmic staining (0, 0 %; 1, <10 %; 2, 11–25 %; 3, 26–50 %; 4, 51–75 %; 5, >75 %) and the intensity of staining (0, no staining; 1, weak staining; 2, intermediate staining; 3, strong staining) were assessed, and averages of the grades were taken. The final staining score was defined as the product of scores for the range and intensity of cytoplasmic staining [15]. All specimens were scored blindly.

CD4/CD8 Lymphocytes Population and Inflammation Score

In five cases of each group immunohistochemistry with monoclonal antibodies to T-cell CD4 and CD8 antigens was performed as previously described [16]. Tissue sections were cut 4-μm thick from routinely processed formalin–fixed and paraffin embedded blocks. The slides were oven-dried overnight at 60 °C. The slides were then put inside the Ventana (Benchmark, USA). The Ventana was activated by loading the pre programmed recipe file for the appropriate antibody. For CD4 we use a polyclonal antibody (Spring, CA, USA), and for CD8 a monoclonal antibody, clone SP16 (DBS, CA, USA). Immunohistochemical staining was performed by the I-view DAB detection kit of Ventana. Dark brown staining was defined as positive and no staining was defined as negative. Staining was scored as follows: 0 (no detectable staining); 1 (1–10 % positive cells); 2 (11–50 %); 3 (51–80%); and

4(more than 80%). In cells with positive staining, the staining was intense and uniform, so intensity was not factored into the scoring. The ratio of CD4/CD8 intraepithelial/mucosal lymphocytes was assessed for ten low-power microscopic fields. Inflammation score was measured according to the Sidney system, and compared between the groups [17]. Sydney score for inflammation is the sum of five criteria: *H. pylori* status, atrophy, intestinal metaplasia, lymphocytic and polymorphonuclear cells infiltration. Every criterion has a score of 0–3 (none exists, mild, moderate, severe), thus the range is 0–15.

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences software 19.0 (SPSS, Inc.). Patient groups were compared using the Pearson chi-square test, Fisher's exact test and Duncan test. *P* values were considered significant when ≤ 0.05.

Results

Patient Characteristics

Ulcer biopsies from 92 patients were included in the final set analysis, including 30 *H. pylori* associated ulcers, 18 NSAID associated, 24 combined *H. pylori*/NSAID and 20 idiopathic, neither associated with *H. pylori* nor NSAID use. Patient characteristics are summarized in Table 1. Forty-four patients were men, with no significant differences between the four groups ($P = 0.24$). Mean age was 66.6 years. Patients with *H. pylori*-associated ulcers not receiving NSAID were significantly younger compared to the other three groups ($\alpha = 0.05$, Duncan test). Only 35 % of patients with *H. pylori*/NSAID negative ulcers were Israeli born, compared to 51.4 % (37/72) in the other groups ($P = 0.19$). A total of 70 % (21/30) of patients with *H. pylori* positive/NSAID negative ulcers were born in Israel, Western Europe or the United States, compared to 53.2 % (33/62) in the other three groups ($P = 0.12$).

Clinical Data

Although significant comorbidities were observed in all groups, 80 % (16/20) of patients with *H. pylori*/NSAID negative ulcers were inpatients at the time of their endoscopy, compared to 45.8 % (33/72) in other groups ($P = 0.007$). Furthermore, idiopathic ulcers were associated with decreased survival where 25 % (5/20) died within 12 months of all causes (the combined causes of gastric pathology and non-related causes), compared to 9.7 % (7/72) in other groups ($P = 0.04$). Patients with *H. pylori*/NSAID negative ulcers more often presented with upper GI bleeding, at 45 % (9/20) compared with 26.4 % (19/72) in the other groups, but this did not reach significance. Subacute and asymptomatic presentations (iron deficiency anemia, weight loss, fecal occult blood and screening) were less common in those with *H. pylori*/NSAID negative ulcers (2/20, 10 %, compared to 34/72, 47.2 %; $P = 0.003$). No difference between groups was observed regarding ulcer number or size. *H. pylori* negative/NSAID positive ulcers were more often located in the gastric body 6/18 (33.3 %) compared with other groups 19/74 (25.7 %), but this did not reach significance. The presence of intestinal metaplasia did not differ between groups (Table 2).

Mucin Staining Scores

MUC 1 protein was strongly expressed on the apical membrane of the glands and mucosal surface epithelial cells. The staining score for the surface epithelium was higher but did not reach significance (Table 3). MUC1 expression was significantly higher for both, superficial epithelium and glands, in patients with *H. pylori*/NSAID negative ulcers than in *H. pylori* positive/NSAID negative ($P < 0.0001$; Fig. 1; Table 4). MUC1 was not expressed in patients with *H. pylori* negative/NSAID positive ulcers, and this finding was statistically significant when compared to the other three groups. MUC 4 expression was not significantly different between the glands and surface epithelium, nor between *H. pylori* positive/NSAID negative and patients with *H. pylori*/NSAID negative ulcers (Fig. 1; Tables 3, 4). MUC 17 protein was strongly expressed on the apical membrane of the mucosal epithelial cells. Some small positive vacuoles were demonstrated within the mucin surface cells. The staining intensity was similar between the foveola and glands (Fig. 1; Table 3). Staining intensity was higher in *H. pylori* positive/NSAID negative than in patients with *H. pylori*/NSAID negative ulcers, foveola and glands alike, with mean scores of 11.50 ± 3.47 versus 6.80 ± 4.02, and 9.61 ± 4.26 versus 7.59 ± 3.26, respectively ($P < 0.0001$). This was a mirror image with MUC 1, i.e. the higher MUC1 (group 4) the lower MUC 17, and vice versa (Fig. 1; Table 4).

Sialic Acid Staining Score

The expression of α2-6 linked sialic acid residues, as stained by SNA lectin, was the same in the surface foveola or the glands (Table 3). Staining intensity was lower in *H. pylori* positive/NSAID negative than in patients with *H. pylori*/NSAID negative ulcers only in the surface epithelium ($P = 0.004$; Fig. 1; Table 4). MUC1 and SNA have similar staining behavior when *H. pylori* positive/NSAID

Table 1 Patient characteristics

Characteristics	HP positive/NSAID negative $N = 30$ (32.6 %)	HP negative/NSAID positive $N = 18$ (19.6 %)	HP positive/NSAID positive $N = 24$ (26.1 %)	HP negative/NSAID negative $N = 20$ (20.8 %)
Age, years [mean (SD, range)]	58.6 (18.58, 18–88)	72.17 (10.44, 56–89)	69.46 (13.08, 24–83)	70.3 (12.64, 42–95)
Gender (male) [*n* (%)]	10 (33)	9 (50)	13 (54.2)	12 (60)
Inpatient [*n* (%)]	11 (36.7)	12 (60)	10 (41.7)	16 (80)
Comorbid disease [*n* (%)]				
ASCVD	3 (10)	12 (66.7)	11 (45.8)	6 (30)
COPD	2 (6.7)	4 (22.2)	2 (8.3)	7 (35)
Diabetes	4 (13.3)	4 (22.2)	12 (50)	6 (30)
Current malignancy	1 (3.3)	2 (11.1)	2 (8.3)	2 (10)
Alcohol abuse	0 (0)	0 (0)	0 (0)	3 (15)
Other significant systemic disease	1 (3.3)[a]	1 (5.6)[b]	1 (4.2)[b]	5 (25)[c]
Total[d]	7 (23.3)	15 (83.3)	19 (79.2)	14 (70)
Primary indication for endoscopy, *n* (%)				
Iron deficiency anemia	9 (30)	5 (27.8)	6 (25)	1 (5)
Epigastric pain/GERD	8 (26.7)	6 (33.3)	4 (16.5)	4 (20)
Upper GI bleeding	7 (23.3)	6 (33.3)	6 (25)	9 (45)
Fecal occult blood	2 (6.7)	0 (0)	1 (4.2)	1 (5)
Weight loss	2 (6.7)	1 (5.6)	5 (20.8)	0 (0)
Screening for gastric cancer	2 (6.7)	0 (0)	0 (0)	0 (0)
Esophageal varices	0 (0)	0 (0)	0 (0)	2 (10)
Vomiting	0 (0)	0 (0)	0 (0)	1 (5)
Other[e]	0 (0)	0 (0)	2 (8.3)	2 (10)
Hemoglobin g/dL [mean (SD, range)]	11.0 (3.22, 3.4–16.7)	10.6 (2.77, 5.5–15.5)	11.0 (2.84, 5.7–14.7)	11.1 (2.70, 6.2–15.3)
Died within 12 months of endoscopy	3 (10)	2 (11.1)	2 (8.3)	5 (25)

HP Helicobacter pylori, *NSAID* non-steroidal anti-inflammatory drugs, *SD* standard deviation, *ASCVD* atherosclerotic cardiovascular disease, *COPD* chronic obstructive pulmonary disease, *GERD* gastro esophageal reflux disease, *IDA* iron deficiency anemia, *FOB* fecal occult blood

[a] Inflammatory bowel disease

[b] Hemodialysis

[c] Inflammatory bowel disease, sepsis, cirrhosis (2 cases), tetraplegia following trauma

[d] Number of patients with comorbidities as listed

[e] Fever unknown origin, gastric outlet obstruction, and surveillance following resection of gastric and esophageal carcinoma (1 case for each indication)

negative and patients with *H. pylori*/NSAID negative ulcers are compared. Both have higher staining score in the foveola and glands in patients with *H. pylori*/NSAID negative ulcers than in *H. pylori* positive/NSAID negative, reaching significance for both in the foveola ($P < 0.0001$ and $P = 0.004$, respectively) but only for MUC1 in the glands ($P < 0.0001$ and $P = 0.457$, respectively). ECA staining intensity was significantly higher in the surface epithelium than in the glands, at 13.49 ± 3.08 versus 5.71 ± 4.72 ($P < 0.0001$). No significant difference was found between *H. pylori* positive/NSAID negative and patients with *H. pylori*/NSAID negative ulcers in the gland or surface epithelium staining score (Fig. 1; Table 4).

Inflammation Score and Lymphocyte Populations

CD positive T cell count was significantly lower in the NSAID positive groups when compared to the *H. pylori* positive groups ($P = 0.009$, Table 5). As expected the Sydney inflammation score was significantly lower in patients with *H. pylori*/NSAID negative ulcers than in *H. pylori* positive/NSAID negative ($P = 0.017$, Table 6).

Discussion

We found that patients with *H. pylori*/NSAID-negative ulcers had multiple comorbidities, were more often

Table 2 Endoscopic findings

Findings	HP positive/NSAID negative $N = 30$ (32.6 %)	HP negative/NSAID positive $N = 18$ (19.6 %)	HP positive/NSAID positive $N = 24$ (26.1 %)	HP negative/NSAID negative $N = 20$ (20.8 %)
Ulcer number (per patient) *n* (%)				
1	21 (70)	10 (55.6)	13 (54.2)	14 (70)
2	4 (13.3)	3 (16.7)	5 (20.8)	3 (15)
≥3	5 (16.7)	5 (27.8)	6 (25)	3 (15)
Ulcer size (*n*)[a]				
5 mm	18	14	27	14
6–10 mm	7	8	10	11
11–20 mm	5	3	4	4
>20 mm	4	0	0	2
Not specified	2	2	2	0
Ulcer location [*n* (%)]				
Antrum	19 (63.3)	10 (55.6)	18 (75)	16 (80)
Body	9 (30)	6 (33.3)	6 (25)	4 (20)
Cardia	2 (6.7)	2 (11.1)	0 (0)	0 (0)
Ulcer HP positive [*n* (%)]	28[b] (93.3)	0 (0)	20 (83.3)	0 (0)
Ulcer IM [*n* (%)]	5 (16.7)	4 (22.2)	3 (16.7)	4 (20)

HP Helicobacter pylori, *IM* intestinal metaplasia

[a] In ten instances the endoscopist reported "ulcer number" as "multiple." Therefore the total number of ulcers (per procedure) is unknown and percentages cannot be calculated

[b] Cases where *H. pylori* was absent from ulcer margin had either positive rapid urease test, positive C13-urea breath test or positive histology in biopsy not taken from ulcer margin

inpatients at the time of endoscopy, had fewer subacute presentations, and had a poorer survival. This concurs with Chan et al. [18] who noted that three quarters of patients with acutely bleeding *H. pylori*/NSAID-negative ulcers have significant comorbidity including major organ failure and malignancy. A large prospective study found that concomitant diseases and the absence of epigastric pain are independent risk factors for *H. pylori*/NSAID-negative ulcers in the duodenum [19]. A higher number of concomitant diseases were associated with increased ulcer size and depth, and more bleeding complications. Furthermore, our data are consistent with previous findings that idiopathic ulcer is an independent risk factor associated with long-term mortality [20, 21]. In this study, *H. pylori*/NSAID-negative gastric ulcers were associated with underlying systemic disease, which could be severe. This association has been reported previously in idiopathic ulcers [20–22], and suggests the possible role of ischemic or non-specific inflammatory factors in their pathogenesis.

Gastric membrane-bound mucins expression has not been previously studied in the setting of peptic ulcer disease. In the present study, the distribution of immunohistochemical staining for MUC1, MUC4 and MUC17, and lectin binding to representative glycan residues in the margins of gastric peptic ulcer was studied. We compared the staining intensity between four patient groups: *H. pylori* positive, NSAID positive, either positive or both negative. The MUC1 gene is located on chromosome 1q21 and has 1,201 nucleotides. The N-terminal ectodomain of MUC1 consists of variable numbers of 20-amino acid tandem repeats [23]. These sites are subject to O-glycosylation that contributes to a structure that extends beyond the glycocalyx of the cell. The glycan chains are often terminated by sialic acids or sulfate groups. The N terminal of MUC1 is tethered to the cell membrane as a heterodimer with the MUC1 C-terminal subunit, which includes a 58-amino acid extracellular domain, a 28-amino acid transmembrane domain, and a 72-amino acid cytoplasmic tail that contains sites for tyrosine and serine phosphorylation. Other membrane-bound mucins such as MUC4 and MUC17, have a similar structure, sometimes with additional specific domains. MUC17 has two EGF-like domains which flank both sides of a SEA module and precede the transmembrane domain. It has been established that *H. pylori* disrupts the assembly of the mucin molecule via inhibition of galactosyltransferase responsible for synthesis of mucin O-glycans [9, 15]. Furthermore, *H. pylori* reduces gastric mucous viscosity by elevating pH through urease secretion, thereby enhancing its motility within gastric mucous [24]. Kobayashi et al. [25] demonstrated how BabA and SabA adhesins on *H. pylori* bind to Lewis B and sialyl Lewis X (a tetrasaccharide) blood group antigens on MUC5AC,

Table 3 Staining positivity for MUC1, MUC 4 and MUC17 and lectins

Group	MUC1		MUC4		MUC 17		SNA		ECA	
	Surface cell	Gland cell	Surface cell	Gland cell	Surface cell	Gland cell	SURFACE cell	Gland cell	Surface cell	Gland cell
1. HP+/NSAID−	2.93 ± 5.13	2.57 ± 4.50	2.79 ± 4.22	5.50 ± 3.14	11.05 ± 3.65	10.29 ± 4.67	2.00 ± 2.63	2.69 ± 4.01	13.27 ± 3.08	5.62 ± 4.37
	$N = 28$	$N = 28$	$N = 14$	$N = 14$	$N = 21$	$N = 21$	$N = 16$	$N = 16$	$N = 26$	$N = 26$
2. HP-/NSAID +	*0.00 ± 0.00	0.00 ± 0.00	3.38 ± 3.07	3.54 ± 3.82	9.23 ± 3.64	9.57 ± 4.73	5.06 ± 5.52	3.44 ± 4.70	14.07 ± 2.01	4.47 ± 4.32
	$N = 14$	$N = 14$	$N = 13$	$N = 13$	$N = 20$	$N = 20$	$N = 18$	$N = 18$	$N = 15$	$N = 15$
3. HP +/NSAID +	11.00 ± 3.78	7.33 ± 4.13	3.75 ± 2.93	6.44 ± 5.17	5.93 ± 2.99	8.80 ± 3.76	4.18 ± 5.57	6.94 ± 5.30	13.78 ± 2.73	4.61 ± 4.64
	$N = 18$	$N = 18$	$N = 16$	$N = 16$	$N = 19$	$N = 19$	$N = 17$	$N = 17$	$N = 18$	$N = 18$
4. HP-/NSAID−	9.89 ± 4.17	7.63 ± 4.60	4.59 ± 3.77	4.00 ± 4.12	6.93 ± 4.00	8.00 ± 3.48	6.17 ± 4.69	3.94 ± 5.45	13.06 ± 4.06	8.00 ± 5.15
	$N = 19$	$N = 19$	$N = 17$	$N = 17$	$N = 22$	$N = 22$	$N = 18$	$N = 18$	$N = 18$	$N = 18$

HP Helicobacter pylori, NSAID non-steroidal anti-inflammatory drugs

* $P = 0.04$, $P < 0.0001$ and $P < 0.0001$ between group 2 and groups 1, 3, and 4, respectively

facilitating colonization. On the other hand, gastric mucins have antimicrobial properties which are directed against *H. pylori*. Kawakubo et al. [10] demonstrated that unique O-glycans in MUC6 inhibit bacterial biosynthesis of cholesteryl-α-D-glucopyranoside, a major cell wall component. Linden et al. [26] suggest that mucins decorated with Le^b (the binding site for the *H. pylori* BabA adhesin) effectively bind *H. pylori*, thereby impairing its colonization of the mucosal surface.

In the present study, cytoplasmic MUC 17 staining associated with *H. pylori* infection was significantly increased, and was higher at the surface (foveola) and glands areas than in the cases with idiopathic ulcer. The opposite was demonstrated for MUC1 that significantly increased in the foveola and glands in the group of idiopathic ulcer patients. This observation of MUC1 up regulation might be important, since the protection efficiency against acid and pepsin provided by different mucins is probably not equal. The decrease of MUC17 expression in the idiopathic ulcer group, even though partially compensated by higher expression of MUC1, may be insufficient for induction of effective protection. We also found a significant decrease in MUC1 expression in *H. pylori* negative/NSAID positive ulcers. This finding was statistically significant when compared to the other three groups, and cannot easily be explained. We speculate that NSAID therapy decreased MUC1 expression through decrease of prostaglandin E synthesis. The presence of *H. pylori* infection may mask this phenomenon through other causes of mucin synthesis and secretion. Interestingly, foveolar expression of sialic acids in α2-6 glycosidic linkage was significantly higher in these cases, a finding that cannot point towards a specific mucin, but may be a global phenomenon that particularly belongs to idiopathic ulcer disease. The increase in sialic acid residues may affect mucins' protection against aggressive luminal agents. The distance between adjacent carbohydrates side-chains may increase due to the negative charge of sialic acid at the end of each chain, and make the mucin backbone exposed to acid and pepsin.

Helicobacter pylori positive ulcer is associated with a high inflammation rate, thus groups 2 and 4 had significantly lower Sydney inflammation score than groups 1 and 3 ($P = 0.017$, 0.041, 0.029 between groups 1–4, 1–2, and 3–4, respectively). In addition we found a low ratio of T-cell CD4/CD8 in the groups negative for *H. pylori*; but when NSAID was also negative the result did not reach significance. Similar findings were described by Stromberg et al. [27]. In peptic ulcer patients positive for *H. pylori*, the number of intraepithelial T-cell CD4+ was higher than in patients with *H. pylori* infection but without ulcer or in healthy controls negative for *H. pylori*. Thus, *H. pylori* infection recruits CD4+ lymphocytes. These findings are

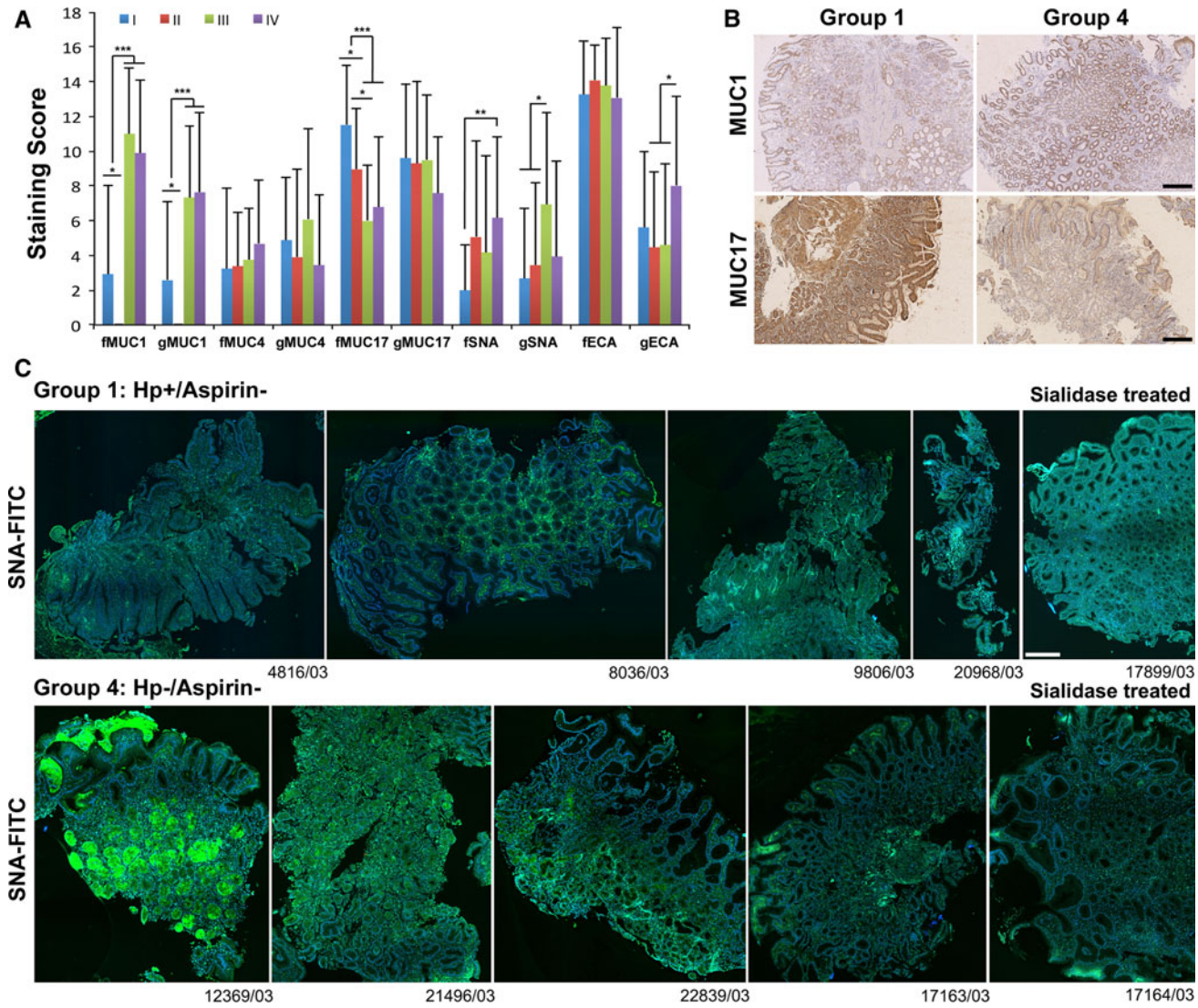

Fig. 1 Staining of different gastric ulcer groups. **a** Staining scores in the different gastric ulcer groups. *f* foveola, *g* glands. I = group 1 = *H. pylori* positive/NSAID negative. II = group 2 = *H. pylori* negative/NSAID positive. III = group 3 = *H. pylori* positive/NSAID positive. IV = group 4 = *H. pylori* positive/NSAID negative. Statistical significance was determined by two-tail *t*-test. $*P < 0.05$; $**P < 0.005$; $***P < 0.0001$. **b** MUC 1 stain is stronger in group 4 compared to group 1, in contrast, MUC 17 stain is stronger in group 1 compared to group 4. **c** SNA-FITC lectin staining of sialic acid in α2-6 glycosidic linkage in four representative gastric ulcer samples. SNA-FITC staining is stronger in group 4 compared to group 1. No staining is observed in sialidase treated tissue, confirming SNA binding specificity. *Scale bars* = 250 micrometers. Staining scores for the different patient groups were compared using the Pearson chi-square test, Fisher's exact test and Duncan test. *P* values were considered significant when ≤ 0.05

also in agreement with Kobayashi et al. [25], describing the high endothelial venule-like vasculature that presumably facilitate lymphocyte recruitment in inflammation sites induced by *H. pylori*.

A limitation of our study is the retrospective nature of the data collection, which precluded elimination of false negative tests for *H. pylori* (probably due to PPI, bismuth or antibiotics), and cases of surreptitious or unreported NSAID use, which would result in misclassification of ulcers as *H. pylori*/NSAID-negative. This could only be overcome using a prospective study design by performing multiple tests *for H. pylori* and assaying serum salicylate and plasma thromboxane, respectively. We only compared expression of mucins at ulcer margin among the groups, and did not compare expression in the background mucosa. Therefore one may speculate that MUC expression is related to secondary changes.

In conclusion, patterns of membrane-bound mucins in *H. pylori*/NSAID-negative ulcers need to be further studied in well-designed, prospective studies, which minimize cross-contamination of groups. Idiopathic peptic ulcers are an increasingly encountered entity, with unique clinical

Table 4 Comparison of mucin and sugar residues staining score between group 1 and group 4

Mucin/sugar	Group 1	*N*	Group 4	*N*	*P*
MUC1 foveola	2.93 ± 5.13	28	9.89 ± 4.17	19	<0.0001
MUC1 glands	2.57 ± 4.50	28	7.63 ± 4.60	19	<0.0001
MUC4 foveola	2.79 ± 4.22	14	4.59 ± 3.77	17	0.221
MUC4 glands	5.50 ± 3.41	14	4.00 ± 4.12	17	0.286
MUC17 foveola	11.50 ± 3.47	21	6.80 ± 4.02	22	<0.0001
MUC17 glands	9.61 ± 4.26	21	7.59 ± 3.26	22	<0.0001
SNA foveola	2.00 ± 2.63	16	6.17 ± 4.69	18	0.004
SNA glands	2.69 ± 4.01	16	3.94 ± 5.45	18	0.457
ECA foveola	13.27 ± 3.08	26	13.06 ± 4.06	18	0.846
ECA glands	5.62 ± 4.37	26	8.00 ± 5.15	18	0.107

Table 5 Intraepithelial/mucosal T-cell populations

Group	*N*	CD4+/HPF	CD8+/HPF	CD4+/CD8+
I. Hp+/NSAID−	5	69.6 ± 38.2*	22.0 ± 4.4**	3.15
II. Hp−/NSAID+	5	8.4 ± 12.5*	14.0 ± 9.4**	0.55
III. Hp+/NSAID+	5	50.0 ± 25.2	26.6 ± 20 ± .9	2.99
IV. Hp−/NSAID−	5	38.0 ± 27.7	29.0 ± 11.4	1.15

* $P = 0.009$, ** $P = 0.052$

Table 6 Inflammation score according to the Sydney system

Group	*N*	*H. pylori*	Atrophy	Intestinal metaplasia	Lymphocytes	PMN	Score
Hp+/NSAID−	5	2.20 ± 0.44	0.60 ± 0.54	0.40 ± 0.54	1.80 ± 0.83	1.20 ± 1.30	6.20 ± 2.48*,**
Hp−/NSAID+	5	0.00 ± 0.00	0.40 ± 0.54	0.60 ± 1.34	1.40 ± 0.89	0.20 ± 0.44	2.60 ± 2.19**
Hp+/NSAID+	5	1.80 ± 1.09	0.80 ± 0.83	0.40 ± 0.89	2.00 ± 0.70	1.40 ± 0.89	6.40 ± 3.13***
Hp−/NSAID−	5	0.00 ± 0.00	0.20 ± 0.44	0.20 ± 0.44	1.40 ± 0.54	0.40 ± 0.54	2.20 ± 1.64*,***

PMN polymorphonuclear cells, *Hp Helicobacter pylori*, *NSAID* non steroidal anti inflammatory drugs

* $P = 0.017$; ** $P = 0.041$; *** $P = 0.029$

and endoscopic features. Future efforts should focus on identifying genetic and epigenetic factors which regulate mucin secretion and mucin glycan modifications in this setting, especially in MUC1 and MUC17, membrane-bound mucins.

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Conflict of interest None declared.

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