Relationship between bile salts, bacterial translocation and duodenal mucosal integrity in functional dyspepsia

Bile salts in functional dyspepsia

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ABSTRACT

Background Functional dyspepsia is a complex disorder, in which multiple mechanisms underlie symptom generation, including impaired duodenal barrier function. Moreover, an altered duodenal bile salt pool was recently discovered in patients with functional dyspepsia. We aimed to evaluate the relationship between bile salts, bacterial translocation and duodenal mucosal permeability in functional dyspepsia.

Methods Duodenal biopsies from patients with functional dyspepsia and healthy volunteers were mounted in Ussing chambers to measure mucosal resistance and bacterial passage in the absence and presence of fluorescein conjugated *Escherichia coli* and glyco-ursodeoxycholic acid exposure. In parallel, duodenal fluid aspirates were collected from patients and bile salts were analyzed.

Key results The transepithelial electrical resistance of duodenal biopsies from patients was lower compared to healthy volunteers ($21.4 \pm 1.3 \Omega \cdot \text{cm}^2$ vs. $24.4 \pm 1.2 \Omega \cdot \text{cm}^2$; $p=0.02$; $N=21$). The ratio of glyco-cholic and glyco-chenodeoxycholic acid to tauro- and glyco-ursodeoxycholic acid correlated positively with TEER in patients. Glyco-ursodeoxycholic acid slightly altered the mucosal resistance, resulting in similar values between patient and healthy biopsies ($22.1 \pm 1.0 \Omega \cdot \text{cm}^2$ vs. $23.0 \pm 1.0 \Omega \cdot \text{cm}^2$; $p=0.5$). Bacterial passage after 120 minutes was lower for patient than for healthy biopsies (0.0 (0.0-681.8) vs. 1684.0 (0.0-4773.0) *E. coli* units; $p=0.02$). Glyco-ursodeoxycholic acid increased bacterial passage in patient biopsies (102.1 (0.0-733.0) vs. 638.9 (280.6-2124.0) *E. coli* units; $p=0.009$). No correlation was found between mucosal resistance and bacterial passage.

Conclusions & inferences Patients with functional dyspepsia displayed decreased duodenal mucosal resistance associated with bile salts, however not associated with bacterial passage *in vitro*. In addition, the hydrophilic bile salt glyco-ursodeoxycholic acid abolished differences in mucosal resistance and bacterial passage between patient and control group.

Key words Bacterial passage & uptake, bile salts, duodenal permeability, functional dyspepsia.

INTRODUCTION

Patients with functional dyspepsia (FD) suffer from recurrent or persistent gastroduodenal symptoms without evidence of a structural disease that is likely to explain the symptoms.¹ Despite a high prevalence in the population, available treatment options are limited and the exact mechanism behind the pathophysiology of FD is still obscure.² FD is most likely a complex disorder, in which symptom generation depends on multiple mechanisms, including gastric sensorimotor dysfunction, duodenal mucosal low-grade immune activation and impaired duodenal barrier function.³

In FD, impaired duodenal barrier function is present as an increased duodenal paracellular permeability and an associated decreased expression of mucosal cell-to-cell adhesion molecules.² It was hypothesized by Vanheel et al. and others that impaired barrier function allows transepithelial passage of luminal antigens, resulting in low-grade inflammation.³ However, mucosal permeability may be influenced by multiple factors, including genetic factors, stress and duodenal luminal factors, such as bile salts.³⁻⁵

Hydrophobic bile salts were shown to impair the small intestinal barrier function and increase the intestinal permeability.⁶⁻⁷ An impaired intestinal barrier function may in turn result in bacterial translocation, which is the passage of bacteria and bacterial products across the intestinal barrier, as reported for colonic biopsies of patients with irritable bowel syndrome (IBS).⁸⁻⁹ In a study by Münch et al., it was shown that hydrophobic bile salts worsen mucosal barrier dysfunction in colonic biopsies of patients with collagenous colitis in remission, resulting in increased bacterial uptake.¹⁰

A duodenal altered bile salt composition and a decreased bile salt concentration was recently discovered by our group in patients with FD.¹¹⁻¹³ We hypothesized that these alterations in the duodenal bile salt pool in FD may contribute to impaired duodenal mucosal integrity, resulting in increased bacterial uptake via the transcellular pathway, eventually leading to low-grade inflammation. Therefore, we aimed to evaluate the relationship between bile salts, bacterial uptake and duodenal mucosal permeability in patients with FD and healthy volunteers (HV).

MATERIALS AND METHODS

Study population

Patients fulfilling Rome IV criteria for FD, who were scheduled for upper gastrointestinal endoscopy with duodenal biopsies, were prospectively recruited from the outpatient clinic of the Department of Gastroenterology at the University Hospitals Leuven. Patients with FD and HV were also recruited via a mailing list. Exclusion criteria were: first-degree relatives with celiac disease, diabetes mellitus, allergy/atopy (eczema, asthma, allergic rhinoconjunctivitis), coagulation disorders/anticoagulant therapy and first-degree relatives with Crohn's disease or type I diabetes mellitus. None of the participants was allowed to take antihistamines, ketotifen, cromoglycate, acetylsalicylates, non-steroidal anti-inflammatory drugs, anticholinergics, theophylline, β 2-agonists, codeine or opioid derivatives and proton pump inhibitors for at least 2 weeks prior to the gastroscopy. Psychosocial co-morbidity was assessed by the patient health questionnaire-9 (PHQ-9) and the visceral sensitivity index (VSI) questionnaire was used to evaluate gastrointestinal-specific anxiety. The dyspepsia symptom score (DSS) was used to score the dyspeptic symptom intensity over the preceding 3 months in patients with FD and HV.¹⁴ These protocols were approved by Leuven University Hospitals Ethics Committee (s56910; 18/12/2015 and s56880; 10/1/2017), and all participants approved and signed the informed consent before inclusion in the studies.

Study 1: Duodenal mucosal integrity, fluid aspiration and bile salt analysis

Upper gastroduodenoscopy was performed by an experienced endoscopist to obtain three biopsy samples from the second part of the duodenum with a standard biopsy forceps (Radial Jaw3, outside diameter 2.2 mm; Boston Scientific, Natick, Massachusetts, USA).³ The biopsy specimens were collected in ice-cold oxygenated Krebs–Ringer bicarbonate buffer and mounted in modified Ussing chambers (Mussler Scientific Instruments, Aachen, Germany), with an exposed tissue area of 1.76mm², following the same protocol as stated by Vanheel et al.³ Mucosal integrity was measured as transepithelial electrical resistance (TEER) and paracellular passage of a 4kDa fluorescein isothiocyanate-labeled dextran (FITC-dx4).³ After the gastroduodenoscopy, duodenal fluids were aspirated via a nasoduodenal catheter at fixed time points before and after a liquid meal

(FortimelEnergy®; Nutricia, Zoetendaal, The Netherlands, 150 kcal per 100 mL with 5.9 g proteins, 18.4 g carbohydrates and 5.8 g lipids, pH6.7) and bile salt concentration was analyzed with liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), as previously reported.^{11, 15} Bile salts were expressed as absolute concentrations (mM) and total bile salts was the molar sum of all measured bile salts.¹¹ Fasting and fed bile salts were calculated as the average of all time points in fasted and fed state.¹¹ Primary bile salts were the molar sum of glyco-cholic acid (GCA), tauro-cholic acid (TCA), glyco-chenodeoxycholic acid (GCDCA) and tauro-chenodeoxycholic acid (TCDCA). Secondary bile salts were the molar sum of glyco-deoxycholic acid (GDCA), tauro-deoxycholic acid (TDCA), glyco-ursodeoxycholic acid (GUDCA) and tauro-ursodeoxycholic acid (TUDCA). The bile salt peak occurred approximately 30 minutes after liquid meal intake and was characterized by the maximal amount of total bile salts postprandially.

Study 2: Evaluation of bacterial uptake

Gastroduodenoscopy was performed to obtain eight duodenal biopsy samples per participant. The biopsies were put in ice-cold oxygenated Krebs–Ringer bicarbonate buffer. The biopsies were mounted in Ussing chambers with an exposed tissue area of 1.76mm² using the technique previously described by Wallon et al.¹⁶ Mucosal and serosal compartments were filled with 3ml 10mM mannitol and 10mM glucose in Krebs-Ringer bicarbonate buffer, respectively.³ Solutions were kept at 37°C, pH 7.4 and continuously carbonated with O₂/CO₂ (95/5%).³ Experiments were performed in open-circuit conditions and started with a 30-min equilibration period to establish a steady state.^{3, 16} TEER was measured for 120 min in all biopsies.¹⁶ After equilibration, chemically killed fluorescein-conjugated *Escherichia coli* K-12 BioParticles (Molecular Probes, Leiden, the Netherlands) were added to the mucosal side at a final concentration of 2.10⁶ *E. coli* units/ml.¹⁷ We have already published that patients with FD have an increased permeability to ions as shown by a reduced TEER and that they have an increased paracellular permeability to FD4. In the present study, we wanted to explore whether the transcellular instead of the paracellular pathway is affected. Larger molecules of 40 KDa and bacteria cross the intestinal epithelium via the transcellular pathway. We selected a fluorescein-conjugated *E. coli* to assess the transcellular uptake. These bacteria were killed with paraformaldehyde which stops

their reproduction but retains antigenicity.¹⁷ Half of the biopsies per participant were also exposed to 1, 2 or 5mM GUDCA (pH 7.8±0.3; MW 449.62; Sigma-Aldrich BVBA, Overijse, Belgium) to evaluate the influence of GUDCA on TEER and bacterial uptake in duodenal biopsies of patients with FD and HV. GUDCA, was diluted in Krebs containing 10mM of mannitol to obtain concentrations of 1mM, 2mM and 5 mM.¹⁰ We analyzed the data concerning the separate (1, 2 and 5mM) and the pooled GUDCA conditions (all data together). At time point 0 and after 120 minutes, 300µL of the serosal compartments were collected and analyzed in a fluorescence reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany) at 480nm excitation and 520nm emission level.¹⁷ We calculated the *E. coli* units from fluorescence intensity using an *E. coli* standard curve. In addition, two biopsies per participant (one exposed to *E. coli* and one exposed to *E. coli* and a certain concentration of GUDCA) were fixated with 4% formaldehyde in phosphate buffered saline (PBS) (pH 7.4) at time points 30, 45 or 120 minutes at 4°C overnight. Afterwards, the biopsies were kept in 30% sucrose for at least 2 days. After fixation, the biopsies were positioned in a cryomold with tissue tek O.C.T. compound (Sakura Finetek Belgium BVBA) and stored at -80°C until sectioning using a cryostat microtome. Sections of 10µm dried overnight at room temperature.¹⁰ Prolong Gold antifade with DAPI was used as mounting medium for nuclear and counterstaining. Images were obtained using an XM10 fluorescence microscope (Olympus, Tokyo, Japan) at 40x magnification.

Statistical analysis

Data were analyzed using GraphPad prism 7 and statistical significance was set at $p < 0.05$. Two-tailed paired t-tests or Wilcoxon signed rank tests, depending on data distribution, were performed to analyze between-group differences. Pearson or spearman correlation coefficient was used, where appropriate, for correlation analysis. Finally, correction for multiple testing (Bonferroni) was performed. Parametric values were presented as mean±SEM, while non-parametric values were presented as median (25–75th interquartile range).

RESULTS

Study 1: Duodenal mucosal integrity, fluid aspiration and bile salt analysis

Study population

Three duodenal biopsies and aspirates were obtained from 18 patients with FD (35.4 ± 3.3 years, 6 men, Table 1). Table 2 summarizes the grading of dyspeptic symptoms. Two patients were subtyped as EPS, seven as PDS and nine belonged to the EPS/PDS overlap group based on Rome IV criteria.¹ Based on previously published criteria, two patients were assumed to have post-infectious FD; 13 patients fulfilled criteria for co-morbid IBS.¹⁸

Correlation between bile salts and duodenal permeability in patients with functional dyspepsia

This patient group showed an average TEER of $20.7 \pm 1.2 \Omega \cdot \text{cm}^2$ and permeability for FITC-dx4 of $35.8 \pm 4.1 \text{ pmol}$. TEER and paracellular passage were negatively correlated ($r = -0.82$; $p < 0.0001$). We evaluated possible correlations between individual bile salts, total bile salts, primary bile salts and secondary bile salts concentrations and paracellular passage and TEER (Table 3). In patients with FD, a positive correlation was found between the ratio of GCA and GCDCA to TUDCA and GUDCA and TEER in the fasted ($r = 0.56$; $p = 0.03$; $N = 15$) and the fed state ($r = 0.61$; $p = 0.02$; $N = 14$). In addition, a positive correlation was found between secondary bile salts and paracellular passage ($r = 0.60$; $p = 0.03$, $N = 13$) during the bile salt peak. However, none of these correlations persisted after Bonferroni correction for multiple testing. Duodenal aspirate data were obtained from 17 patients in the fasting state and from 15 patients in the fed state due to incorrect positioning of the catheter in the duodenum. The different number of patients between the correlation with TEER and with paracellular passage is due to methodological problems when assessing FD4-Dextran permeability in two participants.

Study 2: Evaluation of bacterial uptake and *in vitro* effect of GUDCA

Study population

Eight duodenal biopsies were obtained from 21 patients with FD (42.7 ± 3.2 years, 6 men) and 21 HV (38.8 ± 2.9 years, 6 men) (Table 4). Table 5 summarizes the grading of dyspeptic symptoms. Three patients were subtyped as EPS, eight as PDS and nine belonged to the EPS/PDS overlap group based on

Rome IV criteria.¹ Based on previously published criteria, four patients were assumed to have post-infectious FD; 16 patients fulfilled criteria for co-morbid IBS.¹⁸

Duodenal mucosal epithelial resistance and *E. coli* passage

The duodenal mucosal TEER in biopsies was significantly lower in patients with FD compared to HV ($21.4 \pm 1.3 \Omega \cdot \text{cm}^2$ vs. $24.4 \pm 1.2 \Omega \cdot \text{cm}^2$, $p=0.02$, $N=21$ for each group; Figure 1A). The number of *E. coli* units measured at the basolateral side after 120 minutes was lower in patient biopsies than in HV biopsies (0.0 ($0.0-681.8$) vs. 1684.0 ($0.0-4773.0$) *E. coli* units; $p=0.02$, $N=21$ for each group; Figure 1B).

Glyco-ursodeoxycholic acid abolished the differences in epithelial resistance and *E. coli* passage between patient and control group

Exposure of duodenal biopsies from patients and HV to GUDCA did not significantly alter the TEER ($22.1 \pm 1.0 \Omega \cdot \text{cm}^2$ vs. $21.4 \pm 1.3 \Omega \cdot \text{cm}^2$, $p=0.5$ and $23.0 \pm 1.0 \Omega \cdot \text{cm}^2$ vs. $24.4 \pm 1.2 \Omega \cdot \text{cm}^2$, $p=0.1$, respectively; Figure 1A). However, after exposure to GUDCA, the TEER values between patient and HV biopsies were similar ($p=0.5$). In addition, TEER of patient biopsies exposed to GUDCA was similar to TEER of HV biopsies in standard conditions ($p=0.2$). Furthermore, GUDCA exposure increased *E. coli* passage in patient biopsies (102.1 ($0.0-733.0$) vs. 638.9 ($280.6-2124.0$) *E. coli* units; $p=0.009$), while, GUDCA exposure had no effect on *E. coli* passage in HV biopsies (1066.0 ($0.0-4651.0$) vs. 459.2 ($0.0-2100.0$) *E. coli* units; $p=0.74$) (Figure 1B). Patient and HV biopsies with GUDCA exposure showed similar *E. coli* passage after 120 minutes (708.3 ($344.4-1550$) vs. 369.9 ($0-2100$) *E. coli* units; $p=0.28$). In addition, *E. coli* passage in patient biopsies exposed to GUDCA was similar to *E. coli* passage in HV biopsies at baseline ($p=0.7$). Fluorescence microscopy was used to confirm *E. coli* uptake in the epithelial cell layer of patient and HV duodenal mucosal biopsies after 120 minutes exposure to fluorescent bacteria with and without exposure to GUDCA.

Exposure to glyco-ursodeoxycholic acid decreased *E. coli* passage in duodenal biopsies of healthy volunteers

Patient and HV biopsies were exposed to 1mM GUDCA (N=11 for each group), 2mM GUDCA (N=5 for each group) or 5mM GUDCA (N=5 for each group) compared to no GUDCA exposure (N=21 for each group). No concentration-dependent effect of GUDCA was noticed on TEER and *E. coli* passage in patient biopsies (One-way ANOVA $P=0.2$ and $P=0.23$, respectively; Figure 2A, 2C).

However, *E. coli* passage was decreased in HV biopsies after exposure to 1mM GUDCA compared to standard conditions (76.5 (0.0-550.6) vs. 1035.0 (0.0-4617.0) *E. coli* units; $p=0.03$; Figure 2D).

Correlation between duodenal permeability and bacterial passage

Correlation analysis showed only a strong correlation between TEER and *E. coli* passage in duodenal biopsies of HV after exposure to 2mM GUDCA ($r=0.99$; $p=0.01$; $N=4$). However, the sample size of this group was very small and the correlation did not survive correction for multiple testing. Lack of associations with these two parameters is expected since TEER and *E. coli* uptake involve different pathways. TEER is evaluating the permeability of the duodenal epithelium to ions that can cross via the paracellular and transcellular pathway (mediated by channels and transporters). In contrast, *E. coli* uptake is assessing the transcellular pathway that involves endocytosis and exocytosis of bacteria.

DISCUSSION

In this study, we aimed to further unravel the relationship between bile salts, bacterial translocation and duodenal mucosal permeability in patients with FD and HV. First, we observed a correlation between bile salts and duodenal permeability in patients with FD, in which the hydrophilic bile salt GUDCA seems to be involved. Second, GUDCA abolished the differences in duodenal epithelial resistance and bacterial passage between patients with FD and HV. Third, no correlations were found between bacterial passage and duodenal TEER in HV and patients with FD.

In the present study, a positive correlation was found between the ratio of GCA and GCDCA to TUDCA and GUDCA and TEER during fasting and fed state and also between secondary bile salts and paracellular passage during the bile salt peak in patients with FD. In agreement with our hypothesis, these data confirm a correlation between bile salts and duodenal mucosal permeability in patients with FD. Both correlations link increased GUDCA and TUDCA concentrations to increased duodenal permeability in patients with FD. However, these pilot observations of correlations did not survive correction for multiple testing, and therefore replication in a bigger cohort is warranted. Moreover, it has been suggested in the literature that UDCA conjugates play a role in maintaining the intestinal barrier integrity, which is in apparent contradiction to our data.¹⁹ However, the data from the literature were observed in mice and are based on the effect of pharmacological concentrations of exogenous UDCA rather than concentrations of the endogenous bile salts. We tested this particular ratio of GCA and GCDCA to TUDCA and GUDCA based on the alterations seen in the bile salt concentrations of patients with FD compared to HV in a previous study.¹¹

UDCA is a hydrophilic secondary bile salt with anti-apoptotic and anti-inflammatory effects that was suggested to have therapeutic effects on the colonic intestinal barrier in inflammatory bowel disease (IBD).¹⁹⁻²⁰ In addition, UDCA increases the hydrophilicity index of the circulating bile salt pool, stimulates bile secretion and protects against bile salts and cytokine toxicity.²¹ It has already been reported that intestinal microbiota, food and bile play a significant role in indomethacin-induced small intestinal inflammation and barrier dysfunction in rats.²² Several reports showed that oral UDCA intake

(10mg/kg body weight, three or eight days of intake) decreased the severity of small intestinal injury and protects against indomethacin-induced small intestinal inflammation in rats by modulating intestinal barrier dysfunction and reducing oxidative stress.²¹⁻²² However, Uchida et al. reported that UDCA (after seven days of oral intake) worsened indomethacin-induced small intestinal inflammation in rats by increasing inflammatory scores and shortening the small intestinal length.²³ Oral intake of TCDCA, on the other hand, attenuated intestinal inflammation and prevented indomethacin-induced increases in the hydrophobicity index.²³

Our results and the reported effects of UDCA, UDCA conjugates and other bile salts on intestinal permeability and inflammation may differ due to the use of cancer cell lines versus normal cells, different types of tissue (human vs. rat, small intestine vs. colon), *in vitro* vs. *in vivo*, different dosage and duration of treatment and due to alterations in the *in vivo* bile composition.¹⁹ Golden et al. conducted multiple *in vitro* and *in vivo* studies and concluded that UDCA stimulates intestinal epithelial cell migration and protects from LPS-induced intestinal barrier damage via epithelial EGFR, COX-2, and extracellular signal-regulated kinases (ERK) activation.¹⁹ Considering the migratory and anti-inflammatory effects, UDCA may be useful in the treatment of disorders involving small intestinal damage.¹⁹

The duodenal mucosal TEER, a parameter for permeability, was significantly lower in patients with FD compared to HV. These findings confirm the results from Vanheel et al. and Ishigami et al., which reported impaired duodenal barrier function in patients with FD.³⁻⁴ In our study, exposure of duodenal biopsies to GUDCA slightly altered the TEER, resulting in similar TEER values between patient and HV biopsies. This positive effect of GUDCA on duodenal TEER in patient biopsies agrees with the existing literature, showing that UDCA may stabilize membranes.²⁴ Esteves et al. investigated the effects of the hydrophobic bile salt DCA and hydrophilic bile salt UDCA on monolayers and liposomes.²⁴ They showed that DCA caused fluidization and rupture of monolayers and that UDCA may stabilize membranes.²⁴

Contrary to our hypothesis, decreased transmucosal bacterial passage was observed in biopsies from patients with FD compared to HV. Moreover, GUDCA exposure increased bacterial passage through patient biopsies, resulting in a similar bacterial passage as through HV biopsies, indicating the lack of a protective effect on bacterial translocation. According to the literature, bacterial translocation occurs less frequently in the small intestine compared to the large intestine.²⁴⁻²⁵ In a study by Münch et al., exposure of colonic biopsies from patients with collagenous colitis and HV to 100 $\mu\text{mol/L}$ CDCA or DCA in Ussing chambers resulted in an increased *E. coli* K12 uptake in biopsies from patients with collagenous colitis in remission.¹⁰ However, CDCA or DCA had no effect on bacterial uptake in biopsies from patients with active disease and from patients in remission due to therapy.¹⁰ In the present study, we studied the effect of 1, 2 and 5mM of UDCA, a bile salt known for its cytoprotective effect, on duodenal – rather than colonic – biopsies in a different disorder, and all of these aspects may account for the diverging conclusions.

UDCA is available as a therapeutic agent and part of the goal of the study was to explore a potential beneficial mechanism of UDCA in patients with FD. It has been shown that bile salts modulate barrier function via EGFR or reactive oxygen species in Caco-2 cells and that nonpathogenic *E. coli* strains ameliorate barrier function.²⁶⁻²⁹ Münch et al. hypothesized that luminal bile salts may induce colonic inflammation in susceptible hosts due to unknown mechanisms resulting in hypersensitivity to bile salts.¹⁰ Ward et al. suggested that microbial metabolism of UDCA is necessary for the full expression of its protective effects against colonic inflammation, as they indicate that LCA, the primary metabolite of UDCA, is a more potent inhibitor of intestinal inflammatory responses than UDCA.³⁰ Esteves et al. suggested that the exposure of monolayers to a combination of DCA and UDCA had a greater protective effect than individual bile salts.²⁴ Based on the literature and on our own results, we hypothesize that UDCA and its conjugates may work most efficiently *in vivo* when combined with other bile salts and with microbial metabolization of UDCA into LCA for its full protective effect. In the present *ex vivo* study, exposure of duodenal tissue to high concentrations of the cytoprotective bile salt, GUDCA, elevated duodenal bacterial uptake in biopsies from susceptible hosts (patients with FD with an already impaired barrier function), while having no effect on the biopsies from HV. However, this ‘negative’

effect is limited as the bacterial uptake did not exceed the levels of biopsies from HV. The pre-hypothesized protective effect of GUDCA was only observed in the form of a normalized TEER.

Duodenal biopsies were exposed to 1, 2 or 5mM GUDCA for 2 hours. However, this needs to be carefully interpreted, as the sample size of the 2 and 5mM group is small. Furthermore, these concentrations are relatively high, as the physiological range of GUDCA in duodenal fluid of 21 HV was between 0.04 and 0.21mM with an average of 0.1 ± 0.02 mM.¹¹ GUDCA and TUDCA are the least abundant bile salts in human intestinal fluid.¹¹ For more abundant bile salts, such as GCA and GCDCA, concentrations of 1 to 5mM can be physiological concentrations, as bile salt concentrations vary strongly inter- and intra-individually.¹¹

Finally, there seems to be no correlation between duodenal permeability and *E. coli* passage in HV and in patients with FD. These results indicate that duodenal bacterial translocation is not necessarily enhanced in patients with FD and hence this may be no part of the pathophysiological mechanisms of FD, considering the limitations that we were using a duodenal and an FD unspecific, chemically killed bacterium (*E. coli*). It remains to be shown whether bacterial translocation in FD differs in lower parts of the gastrointestinal tract with higher bacterial exposure. Moreover, our analysis was limited to a single inactivated strain of bacteria which may not be relevant in the pathogenesis of FD. Recent emerging data on the duodenal microbiota in FD suggest that potentially disease-specific strains can be investigated. Indeed, in a small preliminary report including nine patients with FD and nine age-, gender- and BMI-matched controls, the duodenal mucosal microbiota were assessed: the predominant genus was *Streptococcus* in both control and patient group; the anaerobic genera *Prevotella*, *Veillonella* and *Actinomyces* were decreased in patients with FD; the community profiles between patient and control group were significantly different. In addition, duodenal mucosal bacterial load correlated negatively with quality of life and more severe symptom responses to the standardized meal correlated positively with mucosal bacterial load. Finally, the total bacterial load correlated negatively with bacterial diversity.³¹ Furthermore, the intestinal microbiome alters bile salt pool composition, which may also alter the bile salt pool size.³² Gut bacteria can perform multiple biotransformations of bile salts, including the hydrolysis of conjugated bile salts by BSH, the 7 α -dehydroxylation of CA into DCA and CDCA

into LCA, 7 β -dehydroxylation of UDCA into LCA and oxidation and epimerization (of OH-groups at position C3, C7 and C12) resulting in isobile acids.³³⁻³⁴

This *ex vivo* study is limited due to the small sample size and the heterogeneous patient group. Other limitations are the fact that we only tested one specific bile salt, a single measure for permeability and one strain of bacteria, which may not be specific for the duodenum, nor for FD and is chemically killed. Also, our data were analyzed pooling the data from the different GUDCA conditions.

In summary, compared to HV, FD patients have decreased duodenal mucosal resistance which is correlated with an increased ratio of secondary over primary bile salts in duodenal aspirates. The decreased mucosal resistance is not associated with increased *E. coli* translocation across the mucosa *in vitro*. Exposure to the hydrophilic bile salt GUDCA abolished the differences in duodenal epithelial resistance and bacterial passage between patients with FD and HV. These reported effects identify the need for more intensive research in the effects of individual as well as combined bile salts on duodenal permeability and bacterial passage and their role in the pathophysiology of FD. For future research, it is interesting to not only use multiple bile salts in this experimental setup, but also living, duodenum and/or FD specific bacteria.

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DB, RF, ÅK, TV, JT and HV designed the research study. JT and HV acquired funding. PA, JDS, HV and JT provided technical services and materials. DB, TV and JT recruited participants. DB and DR performed the research and analyzed data. DB wrote the paper.

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TABLES

Table 1 Demographics and clinical characteristics of the participants from study 1	
Characteristic	Patients with functional dyspepsia
Gender, Female:Male	12:6 (66.7% female)
Age, years (range)	35.4.7±3.3 (18-57)
Dyspeptic symptoms, DSS	10.3±1.1
BMI, kg/m ²	23.0±0.9
Depression, PHQ-9	5.5 (2.75-8.75)
Gastrointestinal-specific anxiety, VSI	33.6±4.4
Data are mean±SEM or median (IQR); N=18 patients. DSS, dyspepsia symptom score; BMI, body mass index; PHQ-9, patient health questionnaire 9; VSI, visceral sensitivity index.	

Table 2 Frequency of severity grading by DSS for each of the nine dyspeptic symptoms in patients with functional dyspepsia from study 1				
Symptom	absent	mild	moderate	severe
Discomfort	4	4	10	0
Postprandial fullness	2	6	10	0
Bloating	2	4	10	2
Epigastric pain	8	3	6	1
Early satiety	5	6	7	0
Nausea	3	6	7	2
Vomiting	17	0	1	0
Belching	6	5	6	1
Epigastric burning	4	10	3	1
N=18 patients. DSS, dyspepsia symptom score.				

Table 4 Demographics and clinical characteristics of the participants from study 2			
Characteristic	HV	Patients with FD	P-value
Gender, Female:Male	15:6 (71% female)	15:6 (71% female)	-
Age, years (range)	38.8±2.9 (22-61)	42.7±3.2 (18-60)	-
Dyspeptic symptoms, DSS	1.0 (0.0-1.5)	12.0(9.3-14.0)	<0.0001
BMI, kg/m ²	23.9±0.7	23.3±0.8	0.5
Depression, PHQ-9	2.0 (0.0-2.8)	8.0 (4.0-11.0)	<0.0001
Gastrointestinal-specific anxiety, VSI	2.0 (0.5-5.5)	33.0 (22.0-45.0)	<0.0001
Data are mean±SEM or median (IQR); N=21 for both groups. HV, healthy volunteers; FD, functional dyspepsia; DSS, dyspepsia symptom score; BMI, body mass index; PHQ-9, patient health questionnaire 9; VSI, visceral sensitivity index.			

Table 5 Frequency of severity grading by DSS for each of the nine dyspeptic symptoms in patients with functional dyspepsia from study 2				
Symptom	absent	mild	moderate	severe
Discomfort	4	4	12	0
Postprandial fullness	1	5	12	2
Bloating	1	3	12	3
Epigastric pain	5	4	11	0
Early satiety	3	7	9	1
Nausea	3	10	6	1
Vomiting	15	4	1	0
Belching	4	10	5	1
Epigastric burning	6	8	6	0
N= 20 patients. DSS, dyspepsia symptom score.				

FIGURE LEGENDS

Figure 1 Glyco-ursodeoxycholic acid increased the mucosal epithelial resistance and the *E. coli* passage in duodenal biopsies of patients with functional dyspepsia. We pooled the different concentrations of GUDCA. (A) The duodenal mucosal TEER was significantly lower in biopsies from patients with FD compared to HV. After exposure to GUDCA, the TEER values between patient and HV biopsies were similar. (B) *E. coli* passage was higher in HV biopsies than in patient biopsies. Exposure to GUDCA increased *E. coli* passage in biopsies of patients with FD. N=21 for both groups. Data are mean $\pm$ SEM; *p<0.05, **p<0.01. GUDCA, glyco-ursodeoxycholic acid; FD, functional dyspepsia; TEER, transepithelial electrical resistance; HV, healthy volunteers.

Figure 2 Exposure to 1mM glyoursodeoxycholic acid decreased *E. coli* passage in duodenal biopsies of healthy volunteers. Patient and HV duodenal biopsies were exposed to 0mM GUDCA (N=21), 1mM GUDCA (N=11), 2mM GUDCA (N=5) or 5mM GUDCA (N=5). (A-B) No effect of GUDCA on TEER in patient and HV biopsies. (C) No effect of GUDCA on *E. coli* passage in patient biopsies. (D) Exposure to 1mM GUDCA decreased *E. coli* passage in HV biopsies. Data are mean $\pm$ SEM; * $p < 0.05$. GUDCA, glyoursodeoxycholic acid; TEER, transepithelial electrical resistance.