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Duodenal implications in the pathophysiology of functional dyspepsia

Role of bile acids

Dorien Beeckmans

Dissertation presented in partial
fulfillment of the requirements for the
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DUODENAL IMPLICATIONS IN THE PATHOPHYSIOLOGY OF FUNCTIONAL DYSPEPSIA: ROLE OF BILE ACIDS

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DUODENAL IMPLICATIONS IN THE PATHOPHYSIOLOGY OF FUNCTIONAL DYSPEPSIA: ROLE OF BILE ACIDS

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LIST OF ABBREVIATIONS

BA	Bile Acid
BABD	Brisbane Aseptic Biopsy Device
BS	Bile Salt
BSH	Bile Salt Hydrolase
BT	Bacterial Translocation
CA	Cholic Acid
cAMP	cyclic Adenosine Monophosphate
CAR	Constitutive Androstane Receptor
CCK	Cholecystokinin
CDCA	Chenodeoxycholic Acid
COX-2	Cyclo-Oxygenase 2
DCA	Deoxycholic Acid
DMEM	Dulbecco's Modified Eagle's Medium
DSS	Dyspepsia Symptom Score
DSS	Dextran Sodium Sulfate
EGFR	Epidermal Growth Factor Receptor
EPS	Epigastric Pain Syndrome
ERK	Extracellular signal-Regulated Kinases
FaHIF	Fasted Human Intestinal Fluid
FBS	Fetal Bovine Serum
FD	Functional Dyspepsia
FGF19	Fibroblast Growth Factor 19
FGID	Functional Gastrointestinal Disorder
FITC-dx4	Fluorescein Isothiacyanate-labeled dextran 4kDa
fMRI	functional Magnetic Resonance Imaging
FXR	Farnesoid X Receptor
GCA	Glycocholic Acid
GCDCA	Glycochenodeoxycholic Acid
GDCA	Glycodeoxycholic Acid
GERD	Gastroesophageal Reflux Disease
GI	Gastrointestinal
GLP-1	Glucagon-Like Peptide 1
GUDCA	Glycoursodeoxycholic Acid
HBSS	Hanks' Balanced Salt Solution
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethansulfonic acid
HHI	Hydrophilic-Hydrophobic Index
HRM	High Resolution Manometry
HV	Healthy Volunteer
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
Ig	Immunoglobulin
IGP	Intragastric Pressure
IL	Interleukin

LCA	Litocholic Acid
LC-MS/MS	Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
LPDS	Leuven Postprandial Distress Scale diary
LPS	Lipopolysacharide
MAM	Mucosa-Associated-Microbiota
MBP	Major Basic Protein
MES	2-N-Morpholinoethanesulfonic acid
NEAA	Nonessential Amino Acid
NF-κB	Nuclear Factor-κB
*nor*UDCA	*nor*Ursodeoxycholic Acid
NSAID	Nonsteroidal Anti-Inflammatory Drug
PBS	Phosphate Buffered Saline
PCoA	Principal Coordinative Analysis
PDS	Postprandial Distress Syndrome
PHQ-9	Patient Health Questionnaire-9
PI-FD	Post Infectious-Functional Dyspepsia
PPI	Proton Pump Inhibitor
PXR	Pregnane X Receptor
QoL	Quality of Life
SNP	Single Nucleotide Polymorphism
TCA	Taurocholic Acid
TCDCA	Taurochenodeoxycholic Acid
TDCA	Taurodeoxycholic Acid
TEER	Transepithelial Electrical Resistance
TGR5	Transmembrane G-protein Receptor 5
TLCA	Taurolitocholic Acid
TNF	Tumor Necrosis Factor
TRPV1	Transient Receptor Potential cation channel subfamily V member 1
TUDCA	Tauroursodeoxycholic Acid
UDCA	Ursodeoxycholic Acid
VDR	Vitamin D Receptor
VSI	Visceral Sensitivity Index

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Chapter 1

Introduction

1 Introduction

1.1 Functional dyspepsia

Functional gastrointestinal disorders (FGIDs) are common chronic gastrointestinal dysfunctions, which are defined by the Rome Committee and divided into 33 disorders affecting adults and 20 disorders affecting children.[1] One of the most common FGIDs is Functional Dyspepsia (FD).[2] FD is a multifactorial illness, defined by the Rome IV criteria as a medical condition that significantly impacts on the daily activities of a patient and is characterized by one or more symptoms, namely postprandial fullness, early satiation, epigastric pain, and epigastric burning that are unexplained after a routine clinical evaluation.[3] Due to a complex, heterogeneous and incompletely understood underlying pathophysiology, no standard treatment is available for FD.[4] Consequently, FD has major societal implications due to its impact on quality of life (QoL), health care costs and productivity at work.[5-6] An impaired QoL is attributed to emotional distress, diet restrictions and decreased energy levels in patients with FD.[7] FD also has a substantial socio-economic impact in terms of high direct and indirect health care costs due to its high prevalence and associated diagnostic investigations and medication use.[6-9]

1.1.1 Prevalence

Gastric functional and motility disorders are frequently encountered in gastroenterology clinical practice.[10] As shown by multiple articles and reviews on the prevalence of dyspeptic symptoms in the general population, FD affects up to 10–25% of the population with an annual incidence of 1%.[1-2, 5, 7-8] The disappearance rate of FD seems to be similar to the incidence rate.[11] Disappearance of FD may reflect a temporary disappearance of dyspeptic symptoms or a true long-lasting remission.[11] FD is more prevalent in women than in men and can not be associated with a specific age group.[12] The correlation between FD and female predominance may be caused by differences in gastrointestinal function, health care access and pain processing in women compared to men.[11] Interestingly, FD often overlaps with other FGIDs, such as Irritable bowel syndrome (IBS).[5] The prevalence of patients affected by IBS and FD simultaneously ranges between 10% and 27%, and may reach up to 30% in the general population.[11] FD also frequently overlaps with nausea and vomiting disorders, heartburn, somatoform disorders and functional non-intestinal diseases.[5, 11]

1.1.2 Symptom pattern

Patients with FD present with postprandial fullness, early satiation, epigastric pain or burning, upper abdominal bloating, bothersome belching, nausea and vomiting.[10] The Rome III consensus branched FD into two subgroups: the epigastric pain syndrome (EPS) defined by epigastric pain and/or epigastric burning and the postprandial distress syndrome (PDS) defined by meal-induced dyspeptic symptoms, namely postprandial fullness and/or early satiation.[5, 7] Nonetheless, there is considerable overlap between EPS and PDS symptoms in the FD population.[5] Subsequently, the Rome IV consensus, published in 2016, defined FD similar to Rome III, but added postprandially occurring pain, nausea and belching to the PDS subgroup to lessen the overlap between PDS and EPS.[10]

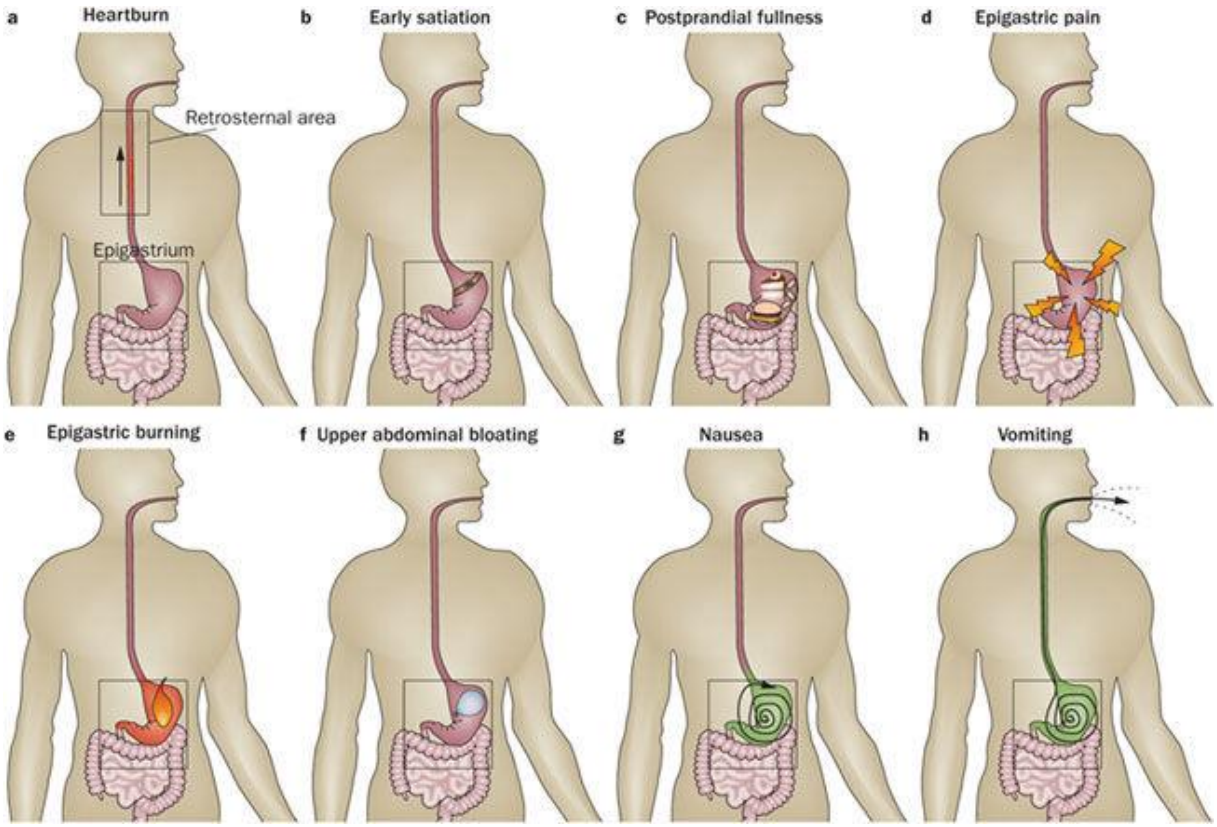


Figure 1.1 Functional dyspepsia symptoms: (A) heartburn, (B) early satiation, (C) postprandial fullness, (D) epigastric pain, (E) epigastric burning, (F) upper abdominal bloating, (G) nausea, (H) vomiting.[13] From Tack et al, 2013.

1.2 Pathophysiological mechanisms

As mentioned before, FD is a complex, multifactorial disorder of which the pathophysiology is not yet fully unravelled.[11] In the following paragraphs, I will describe the pathophysiological and pathogenetic mechanisms which are currently thought to contribute to FD symptom generation, including impaired gastric accommodation, delayed/rapid gastric emptying, gastric hypersensitivity to distention, dietary factors, duodenal hypersensitivity to lipids and acid, low-grade inflammation, impaired duodenal barrier function, acute gastrointestinal infection, dysbiosis, psychosocial comorbidity, altered brain processing, genetic predisposition and stress.[2, 5, 9, 11] I will first review the gastric mechanisms, followed by the duodenal mechanisms and end with the central mechanisms and genetics involved in the pathophysiology of FD.

1.2.1 Gastric mechanisms

Over the last decades, mainly gastric abnormalities were considered to be involved in the pathophysiology of FD.[14] To understand what goes wrong in FD, we need to know how the stomach functions in physiologic conditions. When food enters the stomach, gastric acid and proteolytic enzymes are secreted for the enzymatic digestion of nutrients.[15] The ingested food is initially stored in the proximal stomach.[15] This accommodation reflex is possible by a reduced gastric tone and increased compliance resulting in an increased fundic volume without increase in pressure.[11, 16] Afterwards, the food is advanced to the distal stomach which moulds the ingested meal and coordinates the release of gastric content through the pylorus into the duodenum for further digestion and absorption of nutrients.[14-16] Gastric emptying usually occurs in aliquots, 2–4 times per minute.[15]

1.2.1.1 Impaired gastric accommodation

As mentioned above, gastric accommodation is a reflex that reduces the smooth muscle tone during and after the meal, relaxes the stomach and provides a storage for the ingested food.[11, 14, 16] Gastric accommodation involves a vago-vagal reflex and a nitrergic pathway.[14, 16] Patients with FD are reported to have an impairment of the gastric accommodation of the proximal stomach in response to duodenal distension.[11, 14] Impaired gastric accommodation in FD is characterized by a diminished relaxation of the proximal stomach and an altered distribution of the ingested food which is more predominantly located in the distal stomach.[14] In a study by our group, impaired accommodation showed a prevalence of 36.5% among patients with FD.[17] Impaired gastric motor control can cause altered gastric emptying and intestinal reflex activity as well as increased chemosensitivity or mechanosensitivity.[11] Impaired accommodation was shown to be correlated with early satiety and weight loss.[18] In addition, gastric accommodation was reported to be severely impaired and

correlated with postprandial fullness in patients who developed dyspeptic symptoms after a Nissen fundoplication.[19] The origin of impaired accommodation is unclear, but potential dysfunctions may occur at the level of the vago-vagal reflex pathway, the intrinsic inhibitory innervation or the smooth muscles of the proximal stomach.[14, 16] Impaired gastric accommodation in patients affected by acute onset post infectious-FD (PI-FD) was shown to be due to a nitrergic neuron dysfunction in the stomach.[14, 16] In addition, defective enterogastric and antrofundic reflexes may also lead to an impaired accommodation in patients with FD, resulting in an overload of ingested food in the distal stomach.[14, 18] Finally, distention of a hypersensitive stomach leads to symptom occurrence in patients.[14] Interestingly, the accommodation reflex can also be influenced by psychological factors, such as anxiety.[16]

1.2.1.2 Delayed and rapid gastric emptying

Under physiological conditions, gastric emptying is a harmonious process of repeated tonic and peristaltic contractions that drives the food from the proximal to the distal stomach, moulds the ingested meal and blends it with gastric acid in combination with the opening and closing of the pylorus.[14] Delayed gastric emptying can be generated by an altered or impaired gastric motor function resulting in the prolonged accumulation of solid particles in the stomach.[14] Delayed gastric emptying is considered an important pathophysiological mechanism in FD and has a reported prevalence of 20-50% in FD patients, which is similar in both Rome III-defined subgroups (PDS and EPS).[8, 10, 14, 18] Patients with delayed gastric emptying showed more severe postprandial fullness, nausea and vomiting scores, and a lower QoL.[10, 14, 18] However, not all studies were able to find a correlation between delayed gastric emptying and symptom occurrence.[14, 18] Moreover, in some studies prokinetic drugs were able to induce symptom improvement, but this was not the case in every prokinetic drug trial in FD patients and the relationship between symptom improvement and enhanced emptying is weak.[18] Interestingly, it was found that an equal proportion of patients with FD displayed initial rapid gastric emptying and delayed overall gastric emptying, without a difference in symptoms.[18, 20]

1.2.1.3 Hypersensitivity to gastric distention

Visceral hypersensitivity leading to the increased awareness of visceral events is another putative pathophysiological mechanism in FD.[14] The prevalence (depending on the definition of hypersensitivity as abnormal, allodynia or hyperalgesia) of visceral hypersensitivity ranges between 34%-66% in the FD population, without difference in prevalence between Rome III-based subgroups.[10, 11, 14] Hypersensitivity to gastric distention is more prevalent in women and is correlated with increased symptom severity of belching, postprandial pain and weight loss.[10, 14, 18] In addition,

postprandial sensitivity to gastric distension is shown to be correlated with the severity of postprandial fullness and bloating in FD.[14, 21] The mechanisms causing visceral hypersensitivity in FD may involve abnormal distribution of the meal with antral overload and increased tension mechanoreceptor activation, or intensified processing of gastric afferents at the level of the spinal cord or the brain.[14] Besides gastric hypersensitivity to distention duodenal, jejunal or rectal hypersensitivity to distension may also be present in some patients with FD, suggesting a more general hypersensitivity of the central and/or autonomic nervous systems.[11]

1.2.1.4 Dietary factors

Diet plays an important role in dyspeptic symptom generation in FD. It is, for example, known that the intake of spicy foods is correlated with postprandial fullness scores and that the symptom severity provoked by spicy foods was elevated in female and young patients with FD.[10] Also, a subgroup of patients with FD displays hypersensitivity to oral capsaicin intake, which may be caused by the sensitization of transient receptor potential cation channel subfamily V member 1 (TRPV1).[11, 14] TRPV1 is expressed in the gut wall and is activated by among others capsaicin, evoking a sensation of heat and pain.[11] Besides spices and capsaicin, also the intake of lipids and acid can provoke visceral hypersensitivity in patients with FD (see below). In addition, it was reported that gluten intake in patients with FD is associated with early satiety, an elevated intraepithelial lymphocyte count and a decreased expression of tight junction protein claudin-1.[10] In view of this, nutrient factors may contribute to the generation of low-grade inflammation, impaired duodenal barrier function and dyspeptic symptoms in FD.[10, 22]

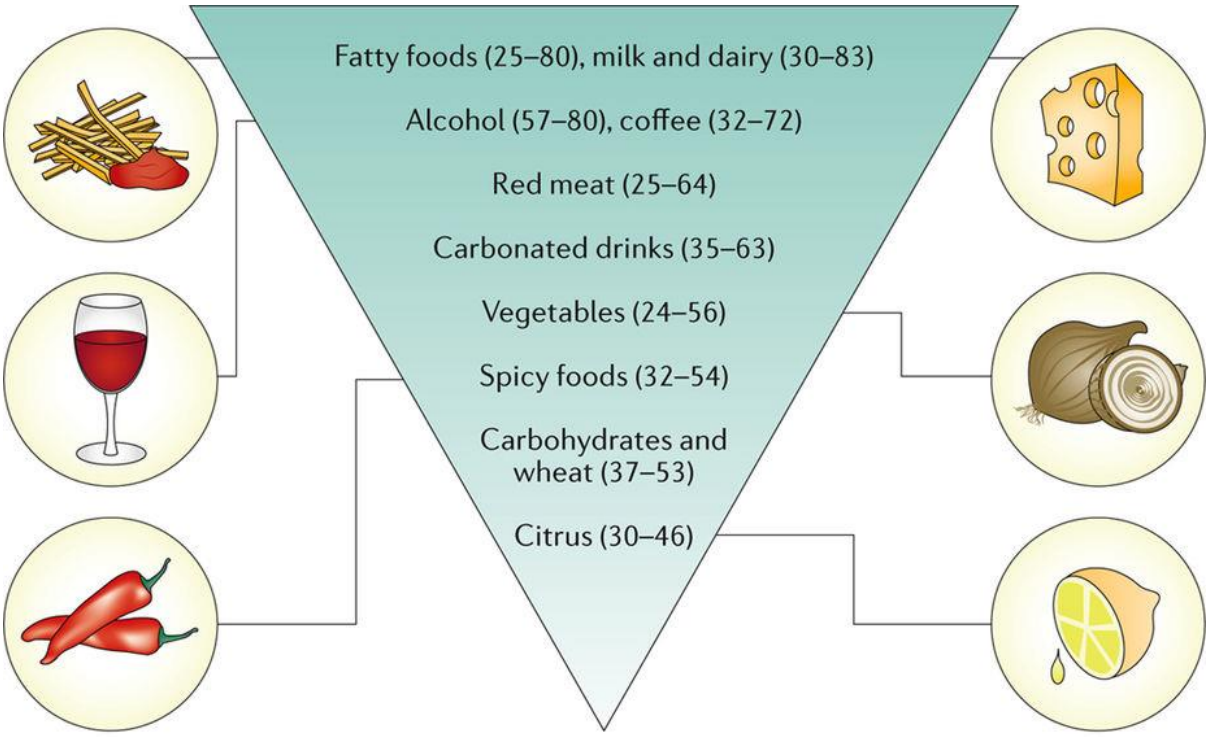


Figure 1.2 Foods possibly evoking onset or worsening of dyspeptic symptoms in patients with functional dyspepsia. The values indicate the percentage of patients reporting an association between a specific food and dyspeptic symptom occurence.[11] From Enck et al, 2017.

1.2.1.5 Gastrointestinal infection

Post infectious-functional dyspepsia

Patients with PI-FD are patients who develop FD after an acute gastrointestinal infection.[14, 23] PI-FD is characterized by the occurrence of impaired accommodation, delayed gastric emptying, early satiety, weight loss, nausea and vomiting.[14] An elevated eosinophil and macrophage count and a correlation between duodenal inflammatory cell infiltration and epigastric burning indicate a role for immune dysfunction in PI-FD.[14] In view of this, it was hypothesized that an acute infection may act as trigger, leading to persisting mucosal inflammation and structural alterations in the upper gastrointestinal tract.[14]

Helicobacter Pylori infection

The best-known upper gastrointestinal pathogen is *H. Pylori* and its role in PI-FD has been a matter of controversy.[24-26] *H. Pylori* infection is a persisting gastric infection and is well correlated with chronic gastritis, gastric and duodenal ulcer occurrence, and risk of gastric cancer and lymphoma.[24-26] It was shown that postprandial gastric and duodenal hypersensitivity is prevalent in *H. Pylori* positive patients with dyspeptic symptoms, although this is not confirmed by all studies.[24, 27] On the other hand, there seems to be no association between *H. Pylori* infection and impaired accommodation or

delayed gastric emptying.[24-25] In addition, it was found that epigastric pain was correlated with *H. Pylori* infection, female sex, age above 40 years and Gastroesophageal Reflux Disease (GERD) symptoms.[25] *H. Pylori* positive patients with FD display increased acid secretion and at least a subset may develop ulcer disease in the long-term.[25] On the other hand, *H. pylori* infection is declining in developed countries as a result of the enhancement of hygiene, living conditions and the environment.[26] However, the *H. pylori* infection prevalence is still around 20% in developed countries and up to almost 100% in some developing countries.[28]

Other infections

Except for *H. Pylori*, the non-invasive protozoan pathogen *Giardia lamblia* may also elicit FGIDs, such as IBS and FD after an acute infection.[29] The underlying mechanism is still unknown, but psychological factors, age, and severity and duration of the acute infection may play a role in the development of FGIDs after an acute gastrointestinal infection.[29]

1.2.2 Duodenal mechanisms

More recent evidence points towards involvement of duodenal abnormalities such as increased sensitivity to duodenal acid/lipids in the pathogenesis of FD symptoms.[14, 30] Early reports also showed the presence of low-grade inflammatory changes in the duodenal mucosa of patients with FD, which has been hypothesized to contribute to symptom generation by inducing altered motor control and visceral hypersensitivity.[14] Moreover, our group recently demonstrated that impaired duodenal barrier function may represent a pathophysiological mechanism in FD, probably related to low-grade duodenal inflammation.[30] These findings state that there are structural changes (not only dysfunctions) and that the duodenum plays a key role in the pathophysiology of FD.[30]

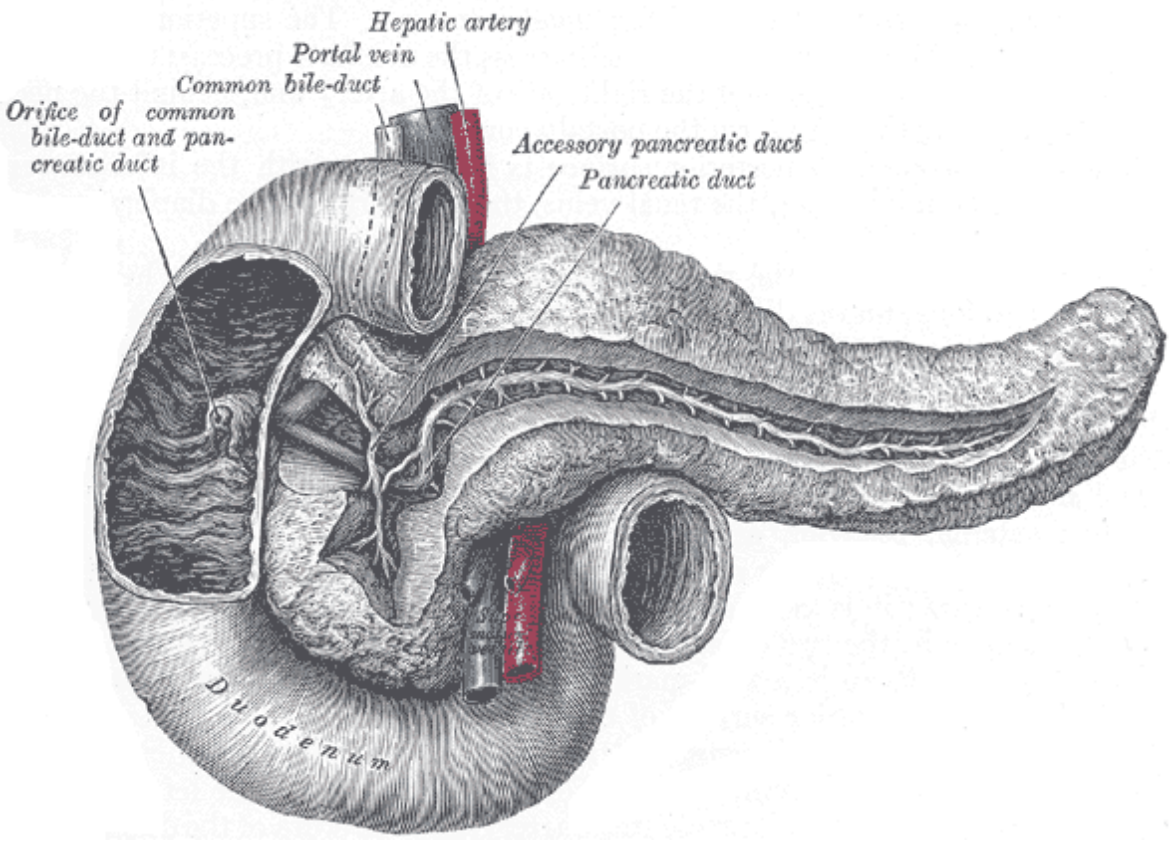


Figure 1.3 Graphical representation of the duodenum and pancreas.[31] From Gray H, 1918.

1.2.2.1 Altered duodenal sensitivity to lipids

It is well established that the majority of patients with FD report an onset or worsening of dyspeptic symptoms after meal intake, more specifically after the intake of fatty meals.[32] A duodenal infusion of a lipid solution in patients with FD and healthy volunteers (HV) resulted in a dose-related induction of hypersensitivity to distention (nausea and bloating) in patients, whereas the volunteers did not report symptom occurrence.[14] These results suggest duodenal hypersensitivity to lipids as another pathophysiological mechanism in FD. Duodenal lipid infusion results in the intensified sensation of gastric distention (epigastric fullness and discomfort) through a cross-sensitization between mechanosensors and chemosensors.[11, 14] Moreover, duodenal lipids can elicit symptoms through neuronal stimulation, lipid hypersensitivity of enteroendocrine cells or nerves, increased levels of systemic or local cholecystokinin (CCK) and/or hypersensitivity to CCK through CCK-A receptors.[11]

1.2.2.2 Altered duodenal sensitivity to acid

Gastroduodenal hypersensitivity to acid has also been reported as a symptom generating mechanism in FD. Acid infusion in patients with FD induces or increases the severity of dyspeptic symptoms (bloating, nausea and satiety) and induces diminished duodenal motor activity and consequently reduced duodenal acid clearance.[14] However, no correlation was found between impaired duodenal

motor activity and hypersensitivity to acid.[14] Moreover, duodenal hypersensitivity and impaired motor function seem to depend upon dysfunction of acid-specific local mucosal chemoreceptors or acid-specific afferent nerves in patients with FD.[14] A subgroup of patients with FD display increased endogenous duodenal acid exposure after meal intake due to reduced duodenal acid clearance.[14] Patients with increased duodenal acid exposure had more severe dyspeptic symptoms than those with normal acid exposure, without correlation between duodenal acidity and symptom severity.[14] It is hypothesized that duodenal acid exposure induces/strengthens/maintains other pathophysiological mechanisms and contributes only indirectly to symptom occurrence. However, another hypothesis is that decreased duodenal acid clearance and symptom generation are both driven by common underlying pathophysiological mechanisms.[14] Furthermore, it was shown that duodenal acidification may play a role in gastric hypersensitivity and impaired motor function through duodeno-gastric reflex pathways.[14] Indeed, duodenal acid regulates gastric emptying, the migrating motor complex phase III, antral pressure waves, gastric accommodation and-, the perception of gastric distention, and may thus be relevant for FD symptom generation.[14]

1.2.2.3 Low-grade inflammation

Duodenal low-grade inflammation is a potentially important pathophysiological mechanism in FD.[5, 11, 14] Low-grade inflammation is evident by the presence of increased duodenal mucosal macrophage and eosinophil counts in patients with FD as a group.[5] The investigation of immune dysfunction in FD was initially triggered by observations of PI-FD (see 1.2.1.5). Patients with PI-FD were characterized by T cell aggregates, decreased amount of CD4+ T cells and elevated numbers of macrophages in the duodenal mucosa.[5, 14] Although less pronounced as in PI-FD, low-grade inflammation also occurs in unspecified-onset (i.e. not established as PI) FD in terms of increased numbers of activated mast cells, a shorter distance of mast cells to nerves and an increased release of histamine, tryptase and serotonin in gastric mucosal biopsies.[5, 14] It was also reported that patients with FD display a systemic immune activation characterized by increased levels of peripheral pro-inflammatory and anti-inflammatory cytokines and gut-homing T cells correlating with pain, cramp, nausea, vomiting severity and delayed gastric emptying.[11, 14] FD patients display duodenal eosinophilia, which is correlated with aberrant nerve structure and defective neuronal responsiveness in the submucosal plexus of the duodenum, which may affect the enteric as well as central nervous systems signalling.[11] This immune activation may contribute to the presence of hypersensitivity to mechanical and chemical factors in FD (see 1.2.1.3 and 1.2.1.4).[11, 14] Moreover, local and circulating cytokines allow cross-talk between the immune system and the gut-brain axis.[11] It was confirmed that patients with FD display a higher number of (degranulated) eosinophils in the duodenum, which correlated positively with postprandial fullness and early satiation and correlated negatively with epigastric pain

frequency.[10] In addition, expression of glial cell line-derived neurotrophic factor, probably indicating a repair mechanism, is significantly increased in FD.[10]

1.2.2.4 Impaired duodenal barrier function

The gastrointestinal barrier is the first defence against pathogens and a selective filter for dietary nutrients, electrolytes and water absorption.[5] This barrier consists of extracellular components, such as bile and mucus, and cellular components, such as epithelial cells.[5] Paracellular permeability, (transport through the space between epithelial cells) is regulated by tight junctions, adherens junctions and desmosomes.[5] These cell-to-cell adhesion proteins determine mucosal permeability.[5] Vanheel et al. reported impaired duodenal barrier function in FD characterized by an increased duodenal permeability, a decreased expression of duodenal mucosal cell-to-cell adhesion proteins and an elevated duodenal eosinophil and mast cell count correlating with impaired duodenal barrier function.[30] A later study confirmed the presence of increased mucosal permeability in all subgroups of FD.[2] This finding upholds the theory of a correlation between impaired duodenal barrier function and low-grade immune activation, however a causal relationship remains to be established.[5, 11, 14, 30] It has been hypothesized that impaired duodenal mucosal integrity allows luminal substances to pass through the mucosa and results in low-grade immune responses.[30] This could provoke visceral hypersensitivity and altered feedback control of gastric motility, finally resulting in dyspeptic symptom generation.[30] Impaired duodenal barrier function can derive from multiple factors, including chronic stress, infection, mast cell activation and cytokines related to allergic or T helper 2 responses.[33] Moreover, serotonin-induced duodenal ion secretion is reduced in FD, which may result in reduced bicarbonate secretion and an impaired protection of the mucosa.[11] Duodenal mucosal integrity is measured as electrical resistance and transmucosal flux of fluorescently labelled macromolecules on biopsies mounted in Ussing chambers.[10] A newly developed method, a catheter-based assessment, measures mucosal admittance directly in patients undergoing an endoscopy.[2, 10] A good correlation was observed between mucosal admittance and mucosal resistance in biopsies.[2] The method seems simple and less invasive than taking biopsies, and may therefore result in a larger scale evaluation of mucosal integrity in FD.[2, 10]

1.2.2.5 Dysbiosis

Role of microbiota

The gut microbiome is the most complex and broadest microbiome in the human body, which plays an important role in the maintenance of body homeostasis and is involved in the induction of metabolic, autoimmune and inflammatory disorders.[26, 34] In view of this, the balance between the gut microbiome and the human body is crucial to human health, since microbiota manage digestive

functions, interplay with the immune system and intestinal epithelial proliferation.[26, 34] The imbalance between the gut microbiome and the human body or dysbiosis may result in disease state.[26, 34] It is reasonable to believe that low-grade inflammation and impaired mucosal integrity can influence the gastrointestinal microbiota, or vice versa.[10] In addition, results from multiple studies suggest a contribution of microbiota to the pathophysiology of FD and suggest a potential role as a therapeutic target.[11] Also, several antimicrobial factors are expressed in the stomach (a defensins, immunoglobulin (ig)A, igG and igM), in the duodenum (b defensins, igA, igG, igM and bile) and in the small intestinal mucosa (collectins and lectins).[26] In the duodenum, bile has direct antibacterial effects.[26]

Several studies have been published on the relationship between gut microbiota and pro- and antibiotic use. It was shown that a two week intake of yogurt containing the *probiotic Lactobacillus Gasseri* in patients with FD enhanced the abundance of gastric *Prevotella* along with symptom improvement.[10, 35] The broad spectrum poorly absorbable antibiotic rifaximin was also reported to improve symptoms of FD.[36] It has been suggested that rifaximin's effect in FD arises partially from its antibiotic effect on the colonic, small intestinal and duodenal microbiota.[36] Acid-suppressive drugs, including proton pump inhibitors (PPIs), are amongst the most commonly prescribed drugs in patients with FD. Use of these acid-suppressive drugs induces hypochlorhydria, changes the natural protection system of the stomach and may alter the gastric and duodenal microbiome.[26] Moreover, a moderate association was found between the risk of gastrointestinal infections and the intake of acid-suppressive drugs.[26] More specifically, PPI use is a risk factor for the development of *Clostridium difficile* infection, small intestinal bacterial overgrowth and adverse outcomes in inflammatory bowel disease (IBD).[37] Furthermore, PPI use increases the presence of *Streptococcaceae* and *Enterococcaceae*, which are risk factors for *C. difficile* infection, and decreases the presence of the commensal anti-inflammatory *Faecalibacterium* in stool samples.[37, 38]

Gastric microbiota

The most widely used approach to investigate the gastrointestinal microbiome is by studying stool microbiota.[28] The bacterial microbiome in patients with gastritis, in patients with cancer and in HV are very much alike, with *Streptococcus* and *Prevotella* as the most abundant genera.[26] This suggests that the stomach is an unfavourable surrounding for microbiota and can exercise selective pressure.[26]

In HV, the most abundant phyla in gastric mucosal biopsies were *Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes* and *Fusobacteria*.[26] Patients with FD display decreased bacterial diversity, a decreased abundance of *Prevotella*, increased abundance of *Bifidobacteria* and *Clostridia*,

a higher abundancy of *Bacteriodetes* compared to *Proteobacteria* and the absence of *Acidobacteria* compared to HV.[10, 11, 35]

Furthermore, it has been shown that systemic inflammation is associated with anxiety and depression in patients with an FGID.[28] The effect of gastric inflammation on the state of mind and mental disorders is not established yet.[28] However, it has been implied that *H. pylori* infection correlates with anxiety indicating that gastric mucosal inflammation can impact on behaviour through the gut-brain axis.[28]

Duodenal microbiota

As changes in GI microbiota have been associated with inflammatory disorders and FGIDS such as IBS, duodenal dysbiosis has been implicated in the pathophysiology of FD.[26, 34] A preliminary report showed that patients with FD display a different duodenal microbiome, with a predominance of species typically found in the oral microbiota, with a positive correlation between total bacterial load and nutrient volume tolerance and an inverse correlation with QoL.[10, 11, 39] Another study found a correlation between *Prevotella* genus and abdominal pain intensity.[11] To colonise the stomach and the duodenum, microbiota need to survive the unfavourable surroundings with barriers of mucus and antimicrobial particles.[26] To improve our understanding about the interplay between the human body and the resident microbiota and their role in health and disease, correct identification of the mucosa-associated microbiota (MAM) is crucial.[34] Until recently, identification of the duodenal microbiome has been inaccurate due to contamination of collected duodenal material by microbiota from mouth, oesophagus and stomach and due to the absence of appropriate methods to identify duodenal microbiota.[26] Stool samples which consist of luminal microbiota, do not allow to identify the MAM.[34] Until now, the collection of duodenal material was generally performed with biopsy forceps. These devices need to pass via an endoscope through the mouth, oesophagus and stomach before reaching the duodenum.[34] Recently, the Brisbane Aseptic Biopsy Device (BABD), an aseptic biopsy device which allows to collect mucosal biopsies without cross-contamination, was developed.[34] This method was shown to be successful in identification of the duodenal MAM, which comprises of *Firmicutes* (>60%; dominant phylum), *Bacteroidetes, Proteobacteria, Actinobacteria*, *Fusobacteria, TM7* and *Acidobacteria* phyla and *Streptococcus* (dominant genus), *Prevotella, Porphyromonas, Veillonella, Fusobacterium, Neisseria, Granulicatella, Leptotrichia* and *Haemophilus* genera with inter-individual variation.[34] The same study also reported that the duodenal microbiota show great overlap with oral microbiota.[34] As mentioned before (1.2.2.3 and 1.2.2.4), patients with FD display duodenal low-grade inflammation, correlating with symptom severity and impaired duodenal barrier function.[30, 34] These characteristics and the existence of PI-FD emphasize the need

to unravel the role of duodenal microbiota and potential immune interactions in the pathophysiology of FD.[34]

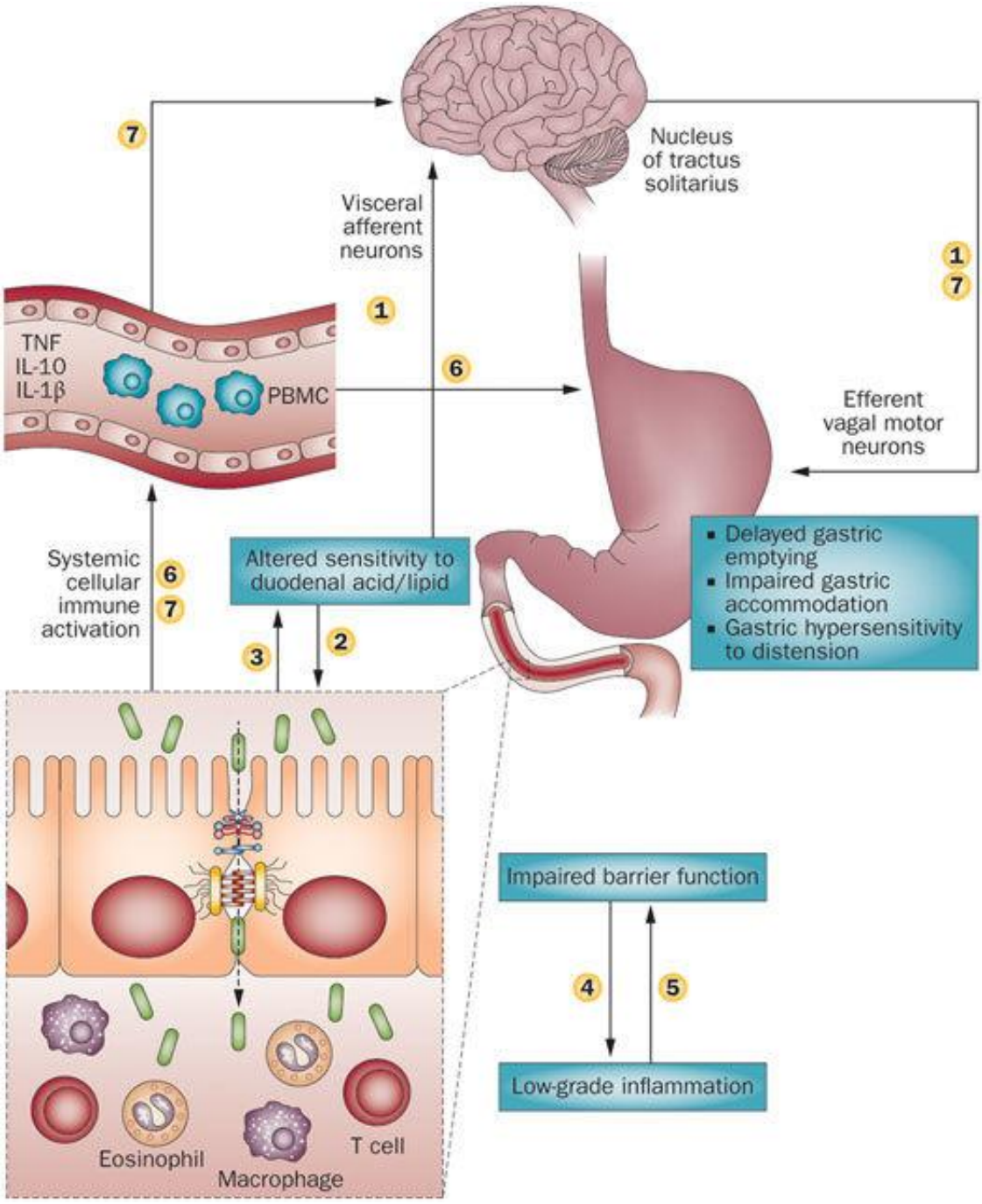


Figure 1.4 Potential mechanisms involved in the pathophysiology of functional dyspepsia.[14] From Vanheel H and Farré R, 2013.

1.2.3 Central mechanisms

1.2.3.1 Psychosocial comorbidity

Psychosocial comorbidities such as anxiety, depression, perceived stress and somatization are suggested to play a role in the pathophysiology of FD, but a causal relationship still needs to be confirmed.[10] Another question not yet fully answered is whether psychosocial comorbidities preceed the onset of FD symptoms or vice versa.[10] Analysis of data from a population-based study showed that the development of gastric dysfunction in patients with mood disorders is preferred by unfavourable socioeconomic elements.[10] As mentioned before, a limited number of patients with FD respond to drug therapy.[28] Anxiety and somatisation are found to be predictors of response to therapy while dysmotility-like symptoms are associated with an adverse response to therapy.[28]

Anxiety and depression

Multiple studies linked anxiety and depression with FGIDs.[28] Anxiety correlates with symptom severity in FGIDs, indicating that affective and cognitive processes are important for the processing of visceral afferents and may play a role in the pathophysiology of FGIDs, including FD.[28] In addition, depression is linked with PDS symptoms, nausea and vomiting in patients with FD.[28]

Stress

Already in 1833, it was noticed that emotional factors can influence gastric functions.[28] Studies in HV demonstrated that acute psychological stress has no impact on gastric acid secretion, but influences the development of interdigestive phase III complexes and pancreatic enzyme secretion.[28] Individual variability determined by personality traits prevails the response to stress.[28] The role of psychological stress on duodenal mucosal barrier function has been studied in HV and showed that acute psychological stress activates the hypothalamo-pituitary-adrenal axis, which in its turn increases duodenal mucosal permeability through corticotropin-releasing hormone and mast cell activation.[5, 40] The role of psychological stress in FD, more specifically impaired duodenal barrier function, needs to be investigated further.[5, 11, 40]

1.2.3.2 Altered brain processing

MRI scans revealed decreased cortical thickness and abnormal brain functional connections in patients with FD, especially in the anterior/posterior cingulate cortex, insula, prefrontal cortex, superior parietal cortex, supramarginal gyrus and the lingual gyrus.[10, 28] These altered brain structures were linked with dyspeptic symptom severity, depression and anxiety scores and disease span, implying that decreased cortical thickness is partially an outcome of FD symptoms rather than a cause.[10, 28] Functional MRI (fMRI) studies reported that the amygdala and the insula are activated

during gastric distension, meaning that both structures are involved in the perception of gastric distention in patients with FD.[28] Also, patients with FD show lower thresholds for discomfort in response to gastric distension and no activation of the periventricular anterior cingulate cortex.[28] The failure of deactivation of the amygdala in patients with FD may reflect arousal-anxiety-driven failure of pain management pathways.[28] Furthermore, childhood abuse is also linked with FGIDs.[28] More specifically, sexual abuse seems to impact on gastric sensitivity and motility.[28] The underlying mechanism may involve neural plasticity as an adaptive brain response in a specific region during childhood, generating behavioural abnormalities during adulthood.[28]

1.2.4 Genetics

Several of the pathophysiological mechanisms in FD described above may be genetically driven. FD is reported to associate with G-protein b polypeptide-3 GNB3 genetic polymorphisms, the GNB3 TT genotype and with the CC genotype of the CCK-A receptor.[10] The homozygous TT CD14rs2569190 genotype, the T allele variant and the heterozygous macrophage migration inhibitory factor GC genotype are more prevalent in patients with FD.[10] On the other hand, the homozygous CC genotype and the C allele of TRPV1 gene are less prevalent in patients with FD.[10] Moreover, CD14 TT genotype is linked with decreased epigastric pain severity, CD14 CT genotype is linked with increased epigastric burning and nausea severity, and macrophage migration inhibitory factor CG is linked with decreased belching severity.[10] No difference is reported between EPS and PDS subgroups.[10]

1.3 Potential pathogenetic factors in functional dyspepsia

Accepted and assumed risk factors for development of FD are gastrointestinal infections (see 1.2.1.5), antibiotic use, intake of nonsteroidal anti-inflammatory drugs (NSAIDs), childhood abuse (see 1.2.4), microbial exposure (see 1.2.2.5), other early life factors, smoking, overweight, stress and psychosocial comorbidities (see 1.2.3).[11] Other factors that potentially underlie dyspeptic symptom generation by impacting the duodenal mucosal barrier and immune activation are genetic factors (see 1.2.4) and luminal factors such as acid (see 1.2.2.2) and bile acids (BA) (see 1.3.1).

It has already been shown that BA may play a role in the pathophysiology of IBS.[41] In addition, BA were shown to impair mucosal integrity in the oesophagus and the colon.[42, 43] Furthermore it is well established that the majority of patients with FD report onset or worsening of symptoms after a meal, which coincides with the release of BA into the duodenum.[32] Finally, changes in the BA pool, provoked by increased duodenal acid exposure, acid-suppressive therapy or an alteration in the microflora, have been shown to increase transmucosal bacterial uptake in colonic biopsies.[43, 44]

1.3.1 Bile acids

BA exert biological effects depending on the chemical structure and the concentration of the BA.[45] This implies that total BA concentration and the BA profile are both relevant when considering BA impact on intestinal functions.[46] Primary BA, cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesized from cholesterol in the liver via the classic or alternative pathway, conjugated with taurine or glycine, stored in the gallbladder forming bile. Bile is secreted postprandially into the duodenum, where BA aid digestion by solubilisation of fat-soluble vitamins and triglycerides due to their amphipathic structure.[47-50] Bile is composed of 85% water and a 15% combination of BA, phospholipids, cholesterol, electrolytes, minerals, proteins, and bilirubin and biliverdin pigments, of the latter generating the yellow-green orange-blue color of bile.[48] The ingestion of lipids results in intestinal secretion of CCK, which stimulates gall bladder contraction and sphincter of Oddi relaxation, leading to duodenal bile secretion.[47-48] More than 90% of BA are reabsorbed by active transport in the terminal ileum (conjugated BA) or by passive transport (nonconjugated BA) throughout the intestinal tract and transported back to the liver via the enterohepatic circulation.[47-49, 51] Less than 10% of the primary BA reach the colon and are either excreted in the stool or metabolized by intestinal microbiota into secondary BA, litocholic acid (LCA) and deoxycholic acid (DCA), which are reabsorbed by the enterohepatic circulation or excreted in the stool.[47-49] Detoxification and efflux systems are present in the colon to protect against these secondary BA.[51] Two to four grams of BA circulate 6-10 times per day in the enterohepatic circulation with a total of 20-40 grams of BA.[48-49] Daily excretion of 200–600 milligrams of BA in the feces needs to be compensated by de novo synthesis from cholesterol in the liver.[47, 48] The BA synthesis is controlled by

positive and negative feedback mechanisms.[47] An important negative feedback mechanism of BA synthesis is controlled by the Farnesoid X receptor (FXR), whose activation results in expression of several genes, including fibroblast growth factor 19 (FGF19).[47] FGF 19 not only inhibits BA synthesis, but also promotes gallbladder refilling, resulting in a decline of circulating BA.[51] Furthermore, FGF19 may play a role in energy homeostasis and probably also has other signalling functions.[51] Without a functioning gallbladder, for example in patients in whom the gallbladder is removed, lipid absorption is still maintained through direct secretion of bile into the duodenum.[48]

Bile acids in in vitro and in vivo experiments

Administration of the secondary BA taurodeoxycholic acid (TDCA) and glycodeoxycholic acid (GDCA) on duodenal brush border membranes of rabbits increases permeability for protons.[52] The primary BA Taurocholic acid (TCA) protects the intestinal barrier by preserving the mucosal integrity and permeability in an obstructive jaundice rat model.[53] Exposure to the secondary BA DCA and trypsin results in decreased transepithelial electrical resistance (TEER) and cell-to-cell adhesion protein expression, such as claudin 3, 4 and E-cadherin, in human esophageal mucosal biopsies.[54] Furthermore, in an ulcerative colitis mouse model, administration of tauroursodeoxycholic acid (TUDCA) results in decreased bacterial translocation (BT), increased mucin production, inhibition of lipopolysacharide (LPS)-induced increased intestinal permeability and inhibition of enterocyte apoptosis.[55] This implicates that TUDCA is a secondary hydrophilic BA with cytoprotective and anti-apoptotic effects.[55] In hepatobiliary diseases with elevated BA serum concentrations, BA may reduce cell mediated immunity and macrophage activation.[48] In obstructive jaundice, Kupffer cell activity is reduced and DCA and CDCA inhibit pro-inflammatory cytokine production (interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α) by macrophages initiated by the presence of bacterial LPS.[48] However, other studies report that CDCA and DCA impair the cell membrane, reduce cholesterol and phospholipid levels, disturb the protection system of the host, induce hydroperoxide production, suppress the immune system and display apoptotic and carcinogenic effects.[45] Moreover, DCA induces colonic epithelium proliferation.[45] Interestingly, the CDCA epimer ursodeoxycholic acid (UDCA) is known for its beneficial effects.[45]

Bile acids in gastroesophageal reflux disease

The gastric BA pool and its pathogenic potential has been studied in detail in GERD. TCA and glycocholic acid (GCA) are the most abundant BA in the refluxate of patients with GERD.[54] During acid suppressive therapy on the other hand, secondary BA become the main BA present in the refluxate of patients with GERD.[54] BA reflux is able to evoke heartburn in patients with GERD and this has been implicated in patients whose symptoms do not respond to PPIs.[47] BA reflux stimulates production of

mucosal IL-8 and cyclo-oxygenase 2 (COX-2), which are involved in gastric and oesophageal metaplasia and carcinogenesis and increase mucosal permeability resulting in mucosal injury by peptic acid, pepsin and trypsin.[47] The association between BA and inflammation is complex since activation of BA receptor transmembrane G-protein-coupled receptor 5 (TGR5) has an anti-inflammatory effect.[47, 48]

Bile acids in functional dyspepsia

BA activation of TGR5 in the ileum induces an ileal brake mechanism by delaying gastric emptying and intestinal motility.[47] In view of this, BA may be involved in the pathophysiology of FD via the alteration of gastrointestinal motility.[47] Gastric infusion of GCA in rats stimulates gastric motility after meal intake.[47] On the contrary, administration of cholestyramine in healthy subjects disrupts the enterohepatic BA circulation and results respectively in CCK release, delayed gastric emptying and a lowered appetite.[47] However, the exact relationship between BA and FD, heartburn and postprandial distress is still not well understood.[47]

1.3.1.1 Function

BA have multiple functions and are crucial players in the lipid metabolism.[48] A first important function of BA is mixed micel formation in the duodenum to aid digestion of lipids and absorption of fat-soluble vitamins A, D, E and K.[48] A second function of BA is the solubilization of cholesterol by micel formation in the gall bladder to prevent cholesterol crystallization and gallstone formation.[48] A third function is the flow of bile from liver to gall bladder. Fourth, conversion of cholesterol to BA and BA excretion in the stool is an important mode of cholesterol elimination.[48-49] Fifth, bile containing mucus and IgA are suggested to have a bacteriostatic function that preserves sterility in the biliary tree.[48] Furthermore, BA are important for clearance of toxic particles, metabolic and antimicrobial effects.[51] BA can bind to and activate four nuclear BA receptors and one BA receptor at the cell surface, namely the FXR, the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), the vitamin D receptor (VDR) and the TGR5, respectively.[48] In addition, BA can act like hormones that modify numerous metabolic pathways throughout the human body.[48, 51] Nuclear receptors are intracellular BA sensors.[51] This network of BA receptors maintains the circulating BA pool and restricts intracellular levels of BA to protect against the potentially toxic effects of BA.[51] Interruption of the BA homeostasis is linked with cholestasis, gallstone disease, inflammation, malabsorption of lipids and fat-soluble vitamins, small intestinal bacterial overgrowth, fatty liver disease, insulin resistance, cancer and multiple other disorders.[48, 51]

1.3.1.2 Bile acid receptors

The BA concentrations in the liver, bile ducts, gallbladder and intestine can activate cell surface BA receptors.[48] The sterol-ring structure enables BA to function through nuclear hormonal receptors, exerting metabolic effects.[47]

1.3.1.2.1 Farnesoid X Receptor

FXR is an important nuclear BA receptor, expressed in the ileum, kidney, adrenal glands and in the liver and displays a set of metabolic hepatic actions, such as regenerative actions and reduction of lipogenesis, gluconeogenesis and hepatic inflammation.[47, 56] Among the BAs, CDCA is the strongest agonist and LCA a weak agonist of FXR.[47] Moreover, FXR regulates BA production (via a negative feedback mechanism), BA absorption and BA removal from the liver.[56] FXR stimulation upregulates FGF19, which inhibits CYP7A1 and subsequently reduces hepatic BA neosynthesis and initiates gallbladder dilatation.[47-48, 56] FXR, on the other hand, can stimulate bile secretion, BA absorption and detoxification to protect the liver against BA toxicity.[56] Furthermore, the BA-FXR-FGF19 signalling cascade is suggested to induce mucin secretion and preserve biliary epithelial cells.[56] FXR agonists do not only regulate BA and lipid homeostasis, but also reduce colonic secretion and display an immunoregulatory activity.[47-48] FXR antagonizes certain signalling pathways, such as nuclear factor (NF)-κB, thereby exerting anti-inflammatory actions.[48] FXR activation reduces the production of proinflammatory cytokines, chemokines and their receptors.[48, 56] FXR protects the intestinal mucosa against bacterial overgrowth and damage and reduces inflammation.[48, 56] In addition, the FXR knockout mouse displays increased BT in the intestinal tract.[47] In summary, FXR initiates five hepatoprotective actions, namely controlling BA production via a negative feedback, alteration of bile composition into a less noxious bile, influencing ductular bicarbonate secretion, anti-inflammatory actions in the liver and influencing the gut-liver axis.[56] Finally, it has been shown that intestinal FXR expression is linked with high-fat-induced intestinal barrier dysfunction and with mucosal integrity.[46] Controlled FXR activation by FXR agonists is a potential therapy for and therefore a research target in patients with cholestasis and IBD.[48, 56] FXR interacts also with other nuclear BA receptors.[47]

1.3.1.2.2 Vitamin D receptor

VDR is a nuclear vitamin D3-dependent steroid hormone receptor, involved in mineral and bone homeostasis, cellular growth and differentiation and secondary BA function.[47-48, 56] VDR also displays immunoregulatory (inhibition of proinflammatory cytokine production) and anti-microbial functions (induction of cathelicidin expression).[48, 56] VDR can also induce FGF19.[47] Besides vitamin D3, LCA is a strong VDR ligand, implying the role of VDR as secondary BA sensor.[56] VDR is expressed in the liver

and gall bladder.[56] VDR activation protects against bacterial infection by induction of cathelicidin, an antimicrobial peptide, and assists innate immunity in the biliary tract.[56] VDR activation stimulates BA absorption in the intestine and BA detoxification in the liver.[56] Furthermore, VDR is crucial for mucosal barrier homeostasis through preservation and restoration of intestinal mucosal integrity.[57] Hepatic VDR activation displays anti-inflammatory and anti-fibrotic effects, implying that vitamin D therapy may be beneficial in patients with hepatobilliary diseases.[56] However, the role of VDR in intestinal BA uptake and hepatic BA metabolism and its negative impact on FXR complicates the potential therapeutic use of vitamin D.[56]

1.3.1.2.3 Pregnane X receptor

The hepatic actions of PXR are comparable to FXR, although PXR also performs intestinal anti-inflammatory actions and induces FGF19 after vitamin A stimulation.[47] PXR inhibits the production of interleukins and chemokines and the expression of NF-κB target genes, such as TNF-α.[48] PXR is responsible for detoxification and elimination (by conversion into more hydrophilic and less noxious compounds) of a wide range of potentially noxious endogenous and foreign compounds, such as xenobiotics, BA and bilirubin, which are activating ligands of PXR.[56] PXR can also repress CYP7A1 and induce BA detoxification enzymes, such as CYP3A4.[56] PXR displays anti-cholestatic, anti-fibrotic and anti-inflammatory actions and is crucial for liver regeneration.[56] Lastly, PXR expression can be stimulated by FXR, implying a cross-talk between nuclear BA receptors to protect against potentially noxious BA.[56]

1.3.1.2.4 Constitutive Androstane Receptor

PXR and CAR perform comparable hepatic actions as FXR.[47] Expression of CAR is localized in the liver and intestine and is regulated by the glucocorticoid receptor.[48] Similar to PXR, CAR functions as a biosensor for endogenous and foreign compounds, such as BA, xenobiotics, lipids, glucose, bilirubin and steroids.[48]

1.3.1.2.5 Transmembrane G-protein coupled Receptor 5

The plasma membrane BA receptor TGR5 is a member of the rhodopsin-like subfamily of G-protein coupled receptors.[48] TGR5 is mainly expressed in the gallbladder, ileum and colon.[48] The preferable ligands of TGR5 are the secondary BA DCA and LCA.[47] TGR5 activation results in inhibition of inflammatory cytokine production (TNF-α, IL-1β, IL-6 and IL-8) and in lowering the proinflammatory capacity of macrophages via cyclic adenosine monophosphate (cAMP) production in innate immune cells.[48] In view of this, TGR5 represents a potential therapeutic target in inflammatory disorders, such as atherosclerosis, arthritis and Crohn's disease.[47-48] Activated TGR5 stimulates glucagon-like peptide

1 (GLP-1) secretion by intestinal enterochromaffin cells and enteric neurons, associating BA with glucose metabolism.[47, 51] Consequently, TGR5 ligands are potential therapeutic agents for metabolic syndrome.[47] Mice overexpressing TGR5 display an accelerated colonic transit, increased water secretion and defecation frequency, while the TGR5 knockout mice presented the opposite.[47] In addition, a TGR5 single nucleotide polymorphism (SNP) has been linked with accelerated small bowel transit in patients with IBS-diarrhoea.[47] On the other hand, TGR5 activation by DCA activates an Ileal brake mechanism to improve uptake of nutrients in the small bowel in mice.[47]

1.3.1.3 Hydrophilicity

In the liver, BA are conjugated with taurine or glycine, which increases their hydrophilicity and assists micelle formation in the duodenum.[48] Nonconjugated BA can permeate through membranes by passive transport, while conjugated BA need active transport.[48] The hydrophilicity of a BA compared to CA is presented by the hydrophilic-hydrophobic index (HHI).[48] Hydrophilic BA (HHI < CA), such as UDCA, are hepatoprotective, while hydrophobic BA (HHI > CA), such as LCA or DCA, are hepatotoxic.[48] Primary BA are more hydrophilic than secondary BA.[48]

1.3.1.4 Ursodeoxycholic acid

UDCA is a hydrophilic, potentially cytoprotective, tertiary BA.[58] UDCA is chemically synthesized for therapeutic use for the dissolution of gallstones, in recurrent pancreatitis, primary biliary cirrhosis, and primary sclerosing cholangitis.[59] UDCA is also suggested to be therapeutic in metabolic syndrome and in colon carcinogenesis.[59] UDCA has several favourable actions, which are in part activated through nuclear receptors.[56] UDCA is no ligand of FXR, and only a ligand for PXR after conversion to LCA by intestinal microbiota.[56] However, UDCA binds and activates VDR, resulting in the induction of the anti-microbial cathelicidin and repair of the mineral and bone homeostasis.[56] In an acute cholecystitis guinea pig model, UDCA protected the gallbladder, while CDCA aggravated the abnormal muscle contractility and the oxidative stress.[60] A derivative of UDCA, 24-*nor*ursodeoxycholic acid (*nor*UDCA), displayed anti-cholestatic, anti-fibrotic, and anti-inflammatory effects by inducing detoxification enzymes, basolateral efflux systems and bicarbonate-rich bile, leading to hepatic BA excretion in a biliary fibrosis mouse model.[56] CAR involvement has been considered in the anti-cholestatic effect of *nor*UDCA.[56] UDCA and *nor*UDCA are considered interesting therapeutic targets for cholestasis and metabolic disorders.[56]

High-fat-feeding increased intestinal permeability, FXR and TNF expression and all BA concentrations except for UDCA, which correlated negatively with intestinal permeability.[46] This implies that high-fat-feeding induced increased intestinal permeability may involve a mechanism linked with BA, reduced UDCA levels and an upregulated FXR expression.[46] The oral intake of fat during a meal

decreases the ratio UDCA/DCA.[58] High-fat diet-related physiological concentrations (1-3mM) of DCA disrupted mucosal integrity, while DCA concentrations during a low-fat diet did not alter mucosal integrity.[58] DCA uptake increased intestinal permeability *in vivo.*[58] UDCA (0.6 mM) on the other hand improved DCA induced disruption of the intestinal mucosal integrity.[58] In view of this, the hydrophilic BA, UDCA, is considered to exert a protective impact on the mucosal barrier: UDCA does not increase epithelial permeability *in vitro*, and is even able to correct intestinal barrier dysfunction.[46] UDCA is probably particularly important when it comes to effects of DCA, as UDCA prevents DCA-induced mitochondrial damage.[46] In humans, UDCA therapy is expected to increase the hydrophilicity of the BA pool and subsequently to prevent the membrane damaging and inflammatory actions of secondary BA on the colonic mucosa and to restrain DCA-activated pathways.[59] However, UDCA can be converted into the noxious LCA through BA 7β-dehydroxylation, which is disadvantageous during UDCA therapy.[59] In a caco-2 monolayer experiment was shown that CA, DCA and CDCA, but not UDCA alter intestinal permeability via epidermal growth factor receptor (EGFR) phosphorylation and tight junction redistribution.[45] CDCA causes an altered distribution of occludin, in which EGFR is an essential intermediate.[45]

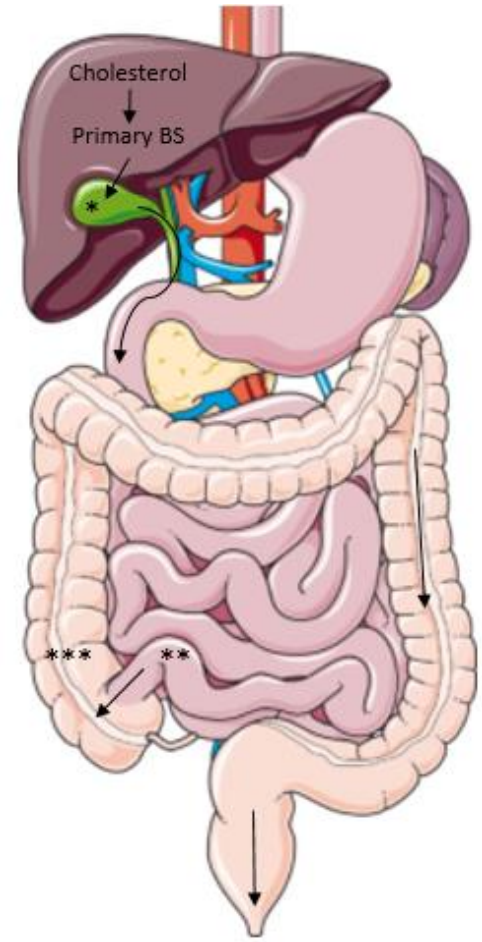


Figure 1.5 Schematic representation of bile acid synthesis and metabolism. * Storage of primary BA (CA and CDCA) conjugated with taurine/glycine in the gall bladder. ** Metabolisation of primary BA into secondary BA (DCA and LCA) by gut microbiota in the terminal ileum. *** Metabolisation of secondary BA into teriary BA (UDCA) by gut microbiota in the colon. Image from https://smart.servier.com/.

1.3.2 The microbiome-bile acid interplay

The gut microbiome affects the composition of the BA pool and vice versa.[48] First of all, gut microbiota can perform multiple BA biotransformations.[59] The most important biotransformations are hydrolysis of conjugated BA into unconjugated BA, hydrolysis of glycine or taurine by bile salt hydrolase (BSH), 7α-dehydroxylation of CA and CDCA creating DCA and LCA, respectively and BA 7β-dehydroxylation of UDCA creating LCA.[59] The BA 7α/7β-dehydroxylations are only performed by anaerobic gut microbiota.[59] The gut microbiota can perform oxidation and epimerization of hydroxy groups at the C3, C7 and C12 position of BA creating isobile (β-hydroxy) acids.[59] Gut microbiota influence BA metabolism by deconjugation and 7α-dehydroxylation of primary BA into secondary BA and influence gene expression managed by FXR.[48] Furthermore, the gut microbiota control expression of multiple enzymes involved in BA synthesis, including CYP7A1.[50] The gut microbiota also control the synthesis of taurine and BA acyl-CoA-synthetase, necessary for BA conjugation.[50]

Microbial deconjugation avoids active small intestinal reabsorption and is performed by microbiota carrying BSH activity.[50] Functional BSH is present in the human gut in members of *lactobacilli, bifidobacteria, Clostridium, Enterococcus* and *Bacteroides* genera, amongst others.[50, 59] UDCA is created by 7a/b-isomerization of CDCA.[50] Furthermore, 3a/b-hydroxy- and 5-H b/a-epimerizations create iso- and allo-BA, respectively.[50] Bacteria with hydroxysteroid dehydrogenases (*Actinobacteria, Proteobacteria, Firmicutes,* and *Bacteroidetes*) catalyse the generation of oxo- (or keto-) BA.[50] BA metabolisation regulated by gut microbiota results in an increased variety and in a more hydrophobic BA pool, which facilitates BA excretion via the faeces.[50] Secondary BA producing bacteria are the *Clostridium* and *Eubacterium* genera from the *Firmicutes* phylum.[50] Second, BA influence the gut microbiota, as they inhibit bacterial endotoxin uptake.[48] Impaired BA production may be a cause of increased bacterial LPS uptake (causing reduced BA excretion and bile flow) and subsequent systemic inflammation in chronic inflammation and autoimmune diseases.[48] Gut microbiota can resist BA via efflux pumps and an alteration of membrane lipid and protein composition.[59] An important antimicrobial mechanism of BA is membrane-damage, which depends on BA concentration and hydrophobicity.[59] Besides membrane-damage, BA can also change the microbial intracellular macromolecular structure via detergent effects, DNA damage and DNA repair enzyme activation.[59] Gram-negative bacteria are more resistant to BA than Gram-positive bacteria.[59] Third, food intake (fat, fiber *et cetera*) also influence the gut microbiota, BA composition and intestinal inflammation.[48]

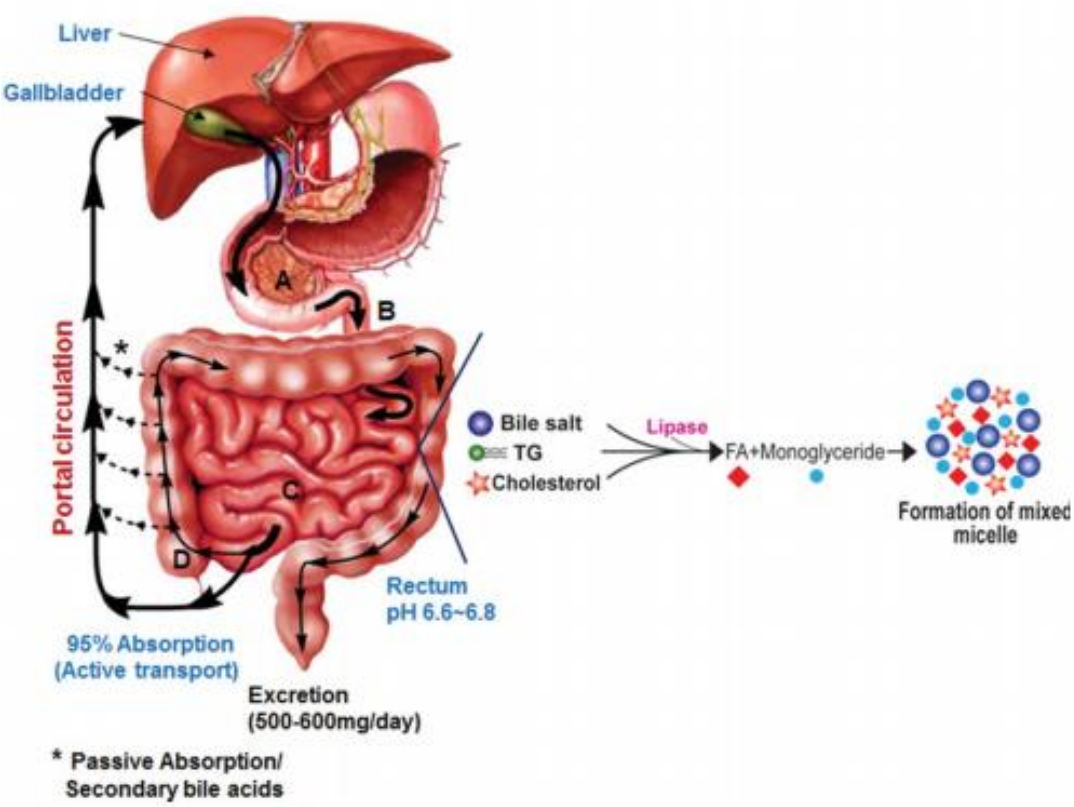


Figure 1.6 The gastrointestinal tract and bile acids in nutrient absorption.[59] From Ridlon et al, 2016.

1.3.3 Vitamin D

As mentioned before, VDR displays immune-modulating, anti-inflammatory, pro-differentiating, and antimitotic effects.[61] Vitamin D can inhibit pro-inflammatory cytokine production of immune cells and macrophages.[61] Furthermore, vitamin D intake may alter the gut microbiota by decreasing the abundance of gammaproteobacteria and increasing bacterial diversity.[61] All in all, vitamin D is a potential therapeutic target in inflammatory (bowel) disorders.[61]

1.4 The intestinal barrier

The intestinal barrier is constantly in touch with antigens derived from food and microorganisms.[62] The function of the intestinal barrier is to control mucosal uptake and to protect against luminal antigens.[62] Under physiological conditions, a limited amount of luminal antigens passes the intestinal barrier and interacts with the host immune system.[62] A disturbed barrier function can result in the passage of an elevated amount of luminal antigens and bacteria, which subsequently can damage the mucosa resulting in pathological conditions.[62] Impaired mucosal integrity is considered to play a role in the pathophysiology of multiple disorders, including FD.[30, 62] However, the cause of intestinal barrier dysfunction is still unknown.[58]

1.4.1 Function

The intestinal barrier is preserved by cellular (interepithelial junctional complexes) and extracellular components (the mucosal surface).[62] The intestinal barrier prevents damage from noxious luminal content, such as LPS and BA.[58] The first line of defence is the presence of luminal gastric acid, pancreatic, and biliary juices degrading bacteria and antigens.[62] Commensal bacteria prevent pathogenic colonization with antimicrobial substances, luminal pH modification and competition for essential nutrients.[62] The micro-environment (the unstirred water layer, glycocalyx and mucus layer with secreted IgA) obstructs the interaction between bacteria and the epithelium.[62] In addition, an epithelial chloride secretion is provoked by noxious stimuli and an antimicrobial peptide secretion destroys microbiota, gathers monocytes and enhances macrophage opsonisation.[62] The intestinal barrier function also is marked by rapid epithelial cell repair and intestinal motility.[62] Intestinal permeability is determined by paracellular (mediated by tight junctions) and transcellular translocation of exogenous molecules.[46]

1.4.2 Structure

The intestinal barrier is a selectively permeable monolayer allowing the uptake of nutrients, electrolytes and water and preventing the uptake of luminal toxins, antigens and gut microbiota.[63] The intestinal barrier is formed by neighbouring epithelial cells connected by complex protein-protein networks, namely desmosomes, adherens junctions and tight junctions.[63] These complex protein-protein networks are transmembrane proteins connecting with epithelial cells and adaptor proteins.[63] The adherens Junctions and desmosomes are important in connecting neighbouring cells, while tight junctions are important for enclosing the intercellular space and controlling paracellular transport.[63] There are multiple transport routes through the intestinal barrier.[62] Passive transport through the intestinal barrier occurs for lipid soluble and small hydrophilic compounds via transcellular diffusion and for medium-sized hydrophilic molecules via paracellular diffusion.[62]

Paracellular transport is impermeable to large molecules and an efficient defence against antigenic macromolecules.[62] Besides passive epithelial absorption, there is also active epithelial uptake.[62] Carrier-mediated uptake can be performed by endocytosis for sugars, amino acids and vitamins, and by transcytosis of endosomal vesicles for larger peptides, proteins and particles.[62] Regulated protein transcytotis is important for antigen control.[62]

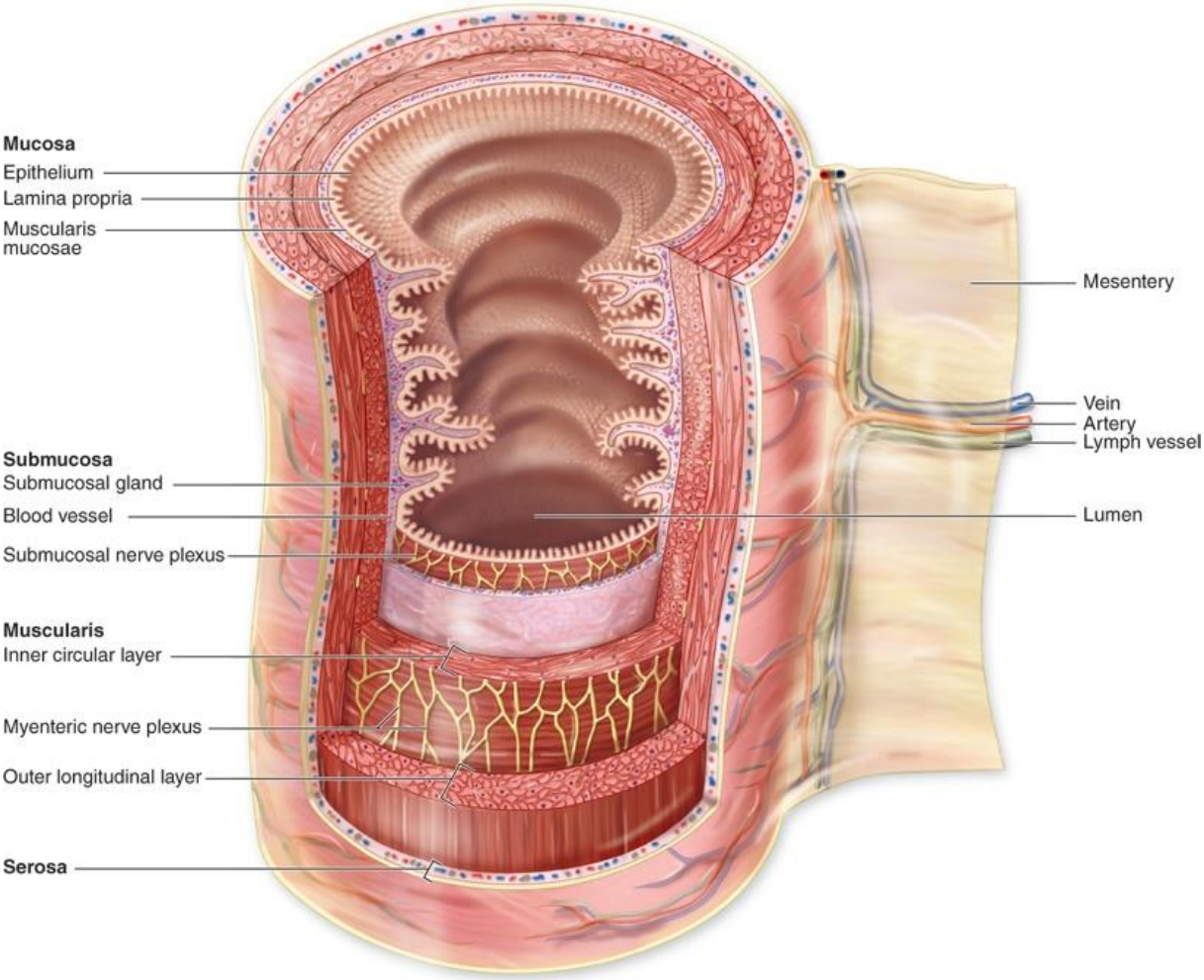


Figure 1.7 Organization of the intestinal tract.[64] From Mescher AL, 2016.

1.4.3 Mucosal integrity

A stable epithelial cell layer is crucial in the preservation of the intestinal barrier.[65] Meaning that the paracellular space between adjacent cells needs to be sealed.[65] The paracellular barrier is formed by the tight junction, adherens junction, and desmosome.[65] Tight junctions are located apicallly and are essential in epithelial transport and in preventing protein and lipid diffusion from the apical to the basolateral side.[62] Adherens junctions are located beneath the tight junctions and both adherens and tight junctions are actin- and myosin-linked membrane complexes.[62, 66] Desmosomes result in interepithelial adhesions and are commonly located beneath the adherens junctions.[62] The adherens

junctions and desmosomes are crucial for cell-cell adhesion, followed by the selective sealing of the intercellular space by the tight junction.[65] The balance between forming a barrier and allowing paracellular transport establishes the function of the gastrointestinal tract.[65] It is known that epithelial cell damage can lead to impaired barrier function.[66] Increasing evidence accumulates on the role of an impaired intestinal paracellular barrier in human diseases, such as IBD.[65]

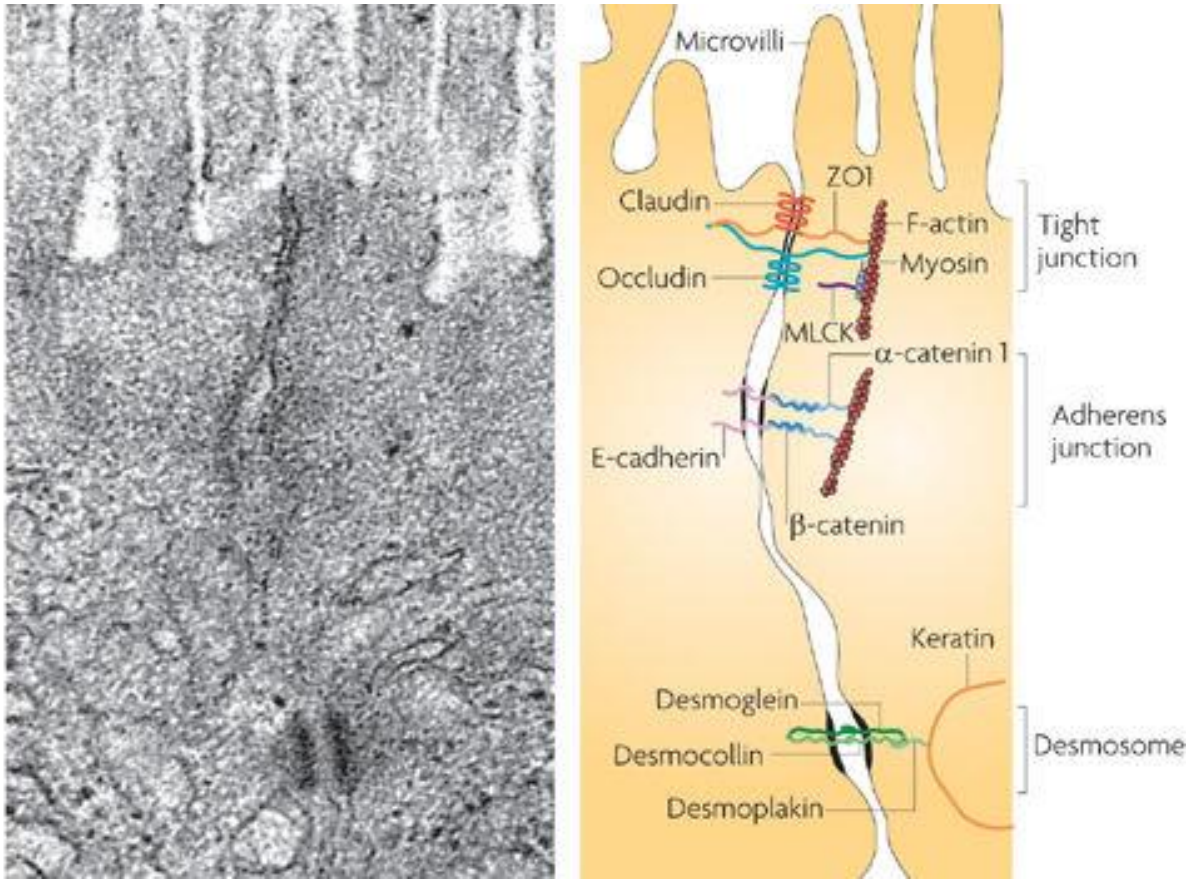


Figure 1.8 Anatomy of the mucosal barrier.[66] From Turner JR, 2009.

Tight junction

The tight junction is a dynamic network of transmembrane, peripheral membrane and regulatory proteins that functions as a selective barrier, assisting the paracellular transport of ions and substances and obstructing the paracellular transport of luminal antigens and microbiota.[63, 66] Tight junctions show four transmembrane protein families: occludin, claudins, junctional adhesion molecules and tricellulin.[63] Important peripheral membrane proteins are zonula occludens 1 and 2.[66] Occludin connects with claudins and actin.[66] Zonula occludens 1 and 2 connect with claudins, occludin and actin and are essential for tight junction aggregation and preservation.[66] Besides involvement in the structure and function of the paracellular barrier, the tight junctions are also involved in cell polarity, signalling, transcriptional regulation, and cell cycle and vesicle trafficking.[67]

The transcellular pathway is less permeable than the paracellular pathway, in which the tight junction restricts solute flux.[66] This implies that the tight junction is the rate-limiting step of paracellular transport and regulates mucosal permeability.[62, 66] There are two tight junction-related transport pathways: the leak pathway and the pore pathway.[66] The leak pathway permits transport of large substances, along with proteins and bacterial LPS but without substances as large as complete bacteria.[66] The leak pathway transport can be influenced by cytokines.[66] The pore pathway is a network of small pores constructed by claudin proteins.[66] Expression of claudins is tissue-specific and can also be altered by cytokines.[66] Increased paracellular permeability can result in epithelial passage of antigens or microbes.[45] This failure of intestinal barrier function is associated with several pathologies.[45]

Adherens junction

Adherens junctions are a network of transmembrane proteins, intracellular adaptor proteins and the cytoskeleton, of which the cadherin-catenin interaction is the most important contributor.[61] Epithelial cadherins are transmembrane glycoproteins that form strong, homotypic connections with molecules on neighboring cells to improve cell-cell adhesion.[63, 66] The catenins, on the other hand, connect the adherens junction to the cytoskeletal network.[63] Moreover, cadherin-catenin complexes also play a role in preservation of cell polarity, management of epithelial migration and proliferation and in the construction of desmosomes.[63] In view of this, a diminished expression of intestinal epithelial cadherin diminishes cell-cell adhesion and is associated with disturbed epithelial proliferation and migration.[63]

Desmosome

Desmosomes are essential for intercellular adhesion and are most abundant in epidermis and myocardium due to the presence of mechanical stress.[68] Their well-organized structure is essential for strong adhesion.[68] Furthermore, desmosomes seem to play a role in cell signalling during development, tissue morphogenesis and wound healing.[68] Desmosomes consist of five important proteins: desmoglein, desmocollin, desmoplakin, plakoglobin and plakophilin.[68]

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Chapter 2

Research objectives

2 Research objectives

The aim of this PhD project is to unravel the pathophysiological role of BA in FD. We hypothesized that increased duodenal BA levels and/or a different BA composition leads to impaired duodenal mucosal integrity in FD, allowing luminal substances to pass through the mucosa, resulting in low-grade immune responses. This could provoke visceral hypersensitivity and altered gastric motor control, finally resulting in dyspeptic symptom generation. On the other hand, increased duodenal permeability could lead to an increased passage of BA, resulting in visceral hypersensitivity and/or altered gastric motor control directly or indirectly via low-grade inflammation.

To accept or reject this hypotheses, we investigated the following research objectives:

1. Characterization of duodenal bile acid composition and receptor expression in functional dyspepsia;
2. Analysis of the relationship between bile acids, bacterial translocation and duodenal mucosal integrity in functional dyspepsia;
3. Evaluation of the effect of ursodeoxycholic acid on duodenal permeability, low-grade inflammation, the gut microbiome and meal related sensory motor function in functional dyspepsia.

2.1 Duodenal bile acid composition and receptor expression in functional dyspepsia

The first objective of this PhD was to evaluate the luminal BA content and BA receptor expression in patients with FD and HV. Furthermore, we evaluated the relationship between luminal BA content and dyspeptic symptom occurrence.

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

The second objective was to obtain knowledge about the influence of BA on duodenal permeability and bacterial uptake in FD. Our aim was to determine whether bacterial uptake occurs in duodenal biopsies of patients with FD compared to duodenal biopsies of HV and whether the BA glycoursodeoxycholic acid (GUDCA) influences mucosal integrity and bacterial uptake of duodenal biopsies from patients with FD and HV in an Ussing chamber system.

2.3 The effect of ursodeoxycholic acid on duodenal permeability, low-grade inflammation, the gut microbiome and meal related sensory motor function in functional dyspepsia

For our last objective, we investigated whether a causal relationship exists between an altered BA pool and increased duodenal permeability, low-grade inflammation, altered gut microbiome, gastric motor control and hypersensitivity.

2.3.1. Effect of ursodeoxycholic acid on duodenal permeability, low-grade inflammation, the gut microbiome and meal related sensory motor function in patients with functional dyspepsia

UDCA is a secondary, hydrophilic BA, used as therapy for several chronic liver disorders such as primary biliary cirrhosis and primary sclerosing cholangitis. It can be used long-term without side effects. Therapeutic properties of UDCA include protection of the liver from cytotoxicity of hydrophobic BA and cytokines by modifying the composition of the BA pool, anti-inflammatory effects and modulation of the intestinal barrier.[1] We aimed to evaluate UDCA as a potential therapy in FD in a mechanistic pilot study. More specifically, we evaluated the effect of UDCA on duodenal permeability, low-grade inflammation, dyspeptic symptoms, BA composition, BA receptor expression, nutrient tolerance, intestinal microbiota composition and intragastric pressure (IGP) in patients with FD.

2.3.2. Effect of ursodeoxycholic acid on mucosal integrity in vitro

According to literature, UDCA can reduce the cytotoxicity of and protect against injuries caused by hydrophobic BA in *in vitro* cell models.[2-3] Hence, our aim was to assess the effect of UDCA on the mucosal integrity, measured as TEER, in Caco-2 cells.

2.4 Note concerning the bile acid/bile salt terminology

Bile salts (BS) are sodium and potassium salts of conjugated BA or in other words BS are the ionized form of the taurine or glycine conjugated BA.[4] Taurine or glycine conjugation increases the amphipathicity and the solubility of BA and leads to impermeability to cell membranes.[4] Ionization at physiological pH, the planar structure and the hydroxyl groups of the conjugated BA increases the amphipathicity of the BS.[4] In the following research chapters, the term ‘bile salt’ would be more appropriate from a chemical perspective, however, we will use the term ‘bile acid’ throughout this thesis manuscript for congruence with everyday terminology, such as UDCA.

2.5 References

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Chapter 3

Altered duodenal bile acid concentration and receptor expression in functional dyspepsia

The data represented in this chapter are published in the following scientific publication:

Beeckmans D, Riethorst D, Augustijns P, Vanuytsel T, Farré R, Tack J and Vanheel H. **Altered duodenal bile salt concentration and receptor expression in functional dyspepsia**. Accepted by UEGJ 31 July 2018.

3 Altered duodenal bile acid composition and receptor expression in functional dyspepsia

3.1 Introduction

FD is a common gastrointestinal disorder defined by the Rome III criteria as the presence of one or more of the following recurrent or persistent symptoms, namely postprandial fullness, early satiation, epigastric pain and epigastric burning.[1] These symptoms originate from the gastroduodenal region without evidence of a structural disease at routine investigations.[1]

Moreover, FD seems to be a heterogeneous disorder in which diverse pathophysiological mechanisms are involved.[2] Over the last few decades, mainly gastric abnormalities have been considered to underlie dyspeptic symptom generation.[2] More recent evidence also points towards involvement of duodenal abnormalities in the pathogenesis of FD.[2] Early reports showed the presence of duodenal mucosal low-grade inflammation in patients with FD.[2] Moreover, impaired duodenal barrier function may represent another pathophysiological mechanism in FD, which is probably related to low-grade inflammation.[3]

The etiology of the latter alterations in FD is unknown but likely candidates are genetic factors, stress and duodenal luminal factors, including BA.[4] BA have been shown to be involved in the pathophysiology of IBS.[5] Furthermore, it is well established that the majority of patients with FD report onset or worsening of symptoms after a meal, which coincides with the release of BA into the duodenum.[6] In addition, increased duodenal acid exposure, acid-suppressive therapy or an alteration in the microbiome, which are often present in FD, can provoke changes in the BA pool.[7] BA can activate five BA receptors, namely TGR5, FXR, PXR, CAR and VDR. These receptors play an important role in diverse processes**,** for instance in the regulation of BA transport and metabolism, inflammation and immunity.[8-9]

We aimed to evaluate the luminal BA content and receptor expression in patients with FD and HV. To this end, we hypothesized that patients with FD display an altered duodenal BA concentration and/or composition or receptor expression, which may contribute to the pathogenesis of FD.

3.2 Materials and methods

3.2.1 Study population

Patients meeting Rome III criteria for FD were recruited from the outpatient clinic of the Department of Gastroenterology at the University Hospitals Leuven or via a mailing list and advertisement.[3] The symptom pattern was confirmed by a questionnaire designed by our group to identify patients with FD according to the Rome III criteria.[10] HV were recruited via a mailing list. Exclusion criteria for all participants were: symptoms or history of gastrointestinal disease (only for HV), celiac disease, diabetes mellitus, allergy/atopy, coagulation disorders/anticoagulant therapy and first degree relatives with celiac disease, Crohn's disease or type I diabetes mellitus.[3] All drugs potentially affecting the BA pool and gastrointestinal permeability were discontinued at least two weeks before the gastroscopy. A Rome III questionnaire was used to evaluate IBS comorbidity. 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 evaluate the dyspeptic symptom intensity over the preceding 3 months in patients with dyspepsia.[11] This protocol conformed to the 1975 Declaration of Helsinki and was approved by Leuven University Hospitals Ethics Committee (s56910; 18/12/2015). All participants approved and signed the informed consent before inclusion in the study.

3.2.2 Experimental methods

3.2.2.1 Western blot

Upper gastroduodenoscopy was performed by an experienced endoscopist to obtain two biopsy samples from the second part of the duodenum with biopsy forceps (Radial Jaw3, outside diameter 2.2 mm; Boston Scientific, Natick, Massachusetts, USA).[3] The biopsy samples were snap-frozen in liquid nitrogen. Protein extracts (15µg) were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane.[3] Membranes were incubated overnight with primary antibodies: rabbit anti-CAR (1:1000; 186869 Abcam, Cambridge, UK), rabbit anti-TGR5 (1:2000; 72608 Abcam), mouse anti-VDR (1:1000; 89626 Abcam), rabbit anti-PXR (1:1000; 85451 Abcam), goat anti-FXR (0.3µg ml^{-1},51970 Abcam). Secondary antibodies used were peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG (both 1:5000; 31460 and 31430, respectively; Thermo Scientific, Leuven, Belgium) or donkey anti-goat IgG (1:5000; 97110 Abcam). As a protein-loading control, all blots were stained with mouse anti-vinculin (1:5000; V9131 Sigma-Aldrich, Overijse, Belgium).[3] Bands were quantified by densitometry using ImageQuant LAS 4000 mini and ImageQuant TL 1D gel analysis software V8.1. Fold change was determined relative to the average of the healthy group.[3]

3.2.2.2 Duodenal fluid aspirations

After the gastroduodenoscopy, an aspiration catheter was placed in the second part of the duodenum under fluoroscopic control. Duodenal fluids were aspirated and collected every 15 minutes during 1 hour in fasted state and 1.5 hours after an oral liquid meal (Fortimel Energy®; 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). Duodenal fluids were centrifuged at 4°C at maximum speed for five minutes and stored at -20°C until BA analysis by a ThermoFisher Scientific liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) system. The pH of every sample was measured with pH-Meter 766 Calimaticon at room temperature (23.5°C). The fasting and fed BA concentration and pH were calculated as the average of all time points in fasted and fed state, respectively. BA were expressed as absolute concentrations (mM) and as percentages of total BA (%). Total BA was the molar sum of all measured BA.

3.2.2.3 Bile acid analysis

Concentration of TCA, GCA, TDCA, GDCA, TUDCA, GUDCA, Taurochenodeoxycholic acid (TCDCA), Glycochenodeoxycholic acid (GCDCA), CA, DCA, CDCA and LCA for each participant and time point was measured with LC-MS/MS by the Drug Delivery and Disposition group (KULeuven, Leuven, Belgium).[12]

3.2.3 Statistical analysis

Data were analyzed using GraphPad prism 7.04 and values were considered significantly different when $p<0.05$. Two-tailed unpaired t-tests or Mann–Whitney U tests, depending on data distribution, were performed to analyze between-group differences. Pearson or spearman correlation coefficient was used, where appropriate, for correlation analysis. Two-way repeated measures ANOVA was used to compare BA concentrations and percentages between groups. Finally, correction for multiple testing was performed with the Bonferroni-correction. Data were presented as mean±SEM or as median (IQR).

3.3 Results

3.3.1 Study population

In total, we recruited 25 patients with FD and 25 HV (Table 3.1). Table 3.2 summarizes the grading of dyspeptic symptoms in the patient group. Six patients were classified as EPS, ten patients as PDS, seven patients belong to the EPS-PDS overlap group and two patients did not fill in the questionnaires. In patients, the PHQ-9 depression score was 5.5 (2.75-9) and the VSI score was 34 (21-45). Based on previously published criteria, two patients were assumed to have post-infectious FD; 19 patients fulfilled criteria for co-morbid IBS.[13] Twelve patients who were taking proton pump inhibitors discontinued the intake at least two weeks prior to the start of the study.

Table 3.1 Demographics and clinical characteristics of the participants

Characteristic	HV	Patients with FD
Gender, Female:Male	12:13 (48% female)	18:7 (72% female)
Age, years (range)	24.6±1.2 (18-45)	**38.3±2.9 (18-57) ***
Dyspeptic symptoms, DSS	1 (0-2)	**11 (8-13) *****
BMI, $kg.m^{-2}$	22.9±0.6	23.5±0.8

Data are mean±SEM or median (IQR); N=25 for both groups. HV, healthy volunteers; FD, functional dyspepsia; DSS, dyspepsia symptom score; BMI, body mass index; *=p<0.05; ***=p<0.0001.

Table 3.2 Frequency of severity grading by DSS for each of the nine dyspeptic symptoms in patients with FD

Symptom	absent	mild	moderate	severe
Discomfort	6	5	13	0
Postprandial fullness	2	9	13	0
Bloating	4	5	13	2
Epigastric pain	9	4	10	1
Early satiety	7	7	10	0
Nausea	6	9	7	2
Vomiting	22	1	1	0
Belching	7	7	8	2
Epigastric burning	6	11	6	1

N=24 patients with FD. DSS, dyspepsia symptom score; FD, functional dyspepsia.

3.3.2 Less acidic duodenal environment after food intake in functional dyspepsia

Duodenal aspirates were obtained from 17 patients and 21 HVs, as the positioning of the catheter in the duodenum was impossible in 12 participants. During fasting, the pH of the duodenal aspirates from patients and HV was similar (pH 6.9±0.3 vs. pH 6.8±0.2; p=0.61). In HV, the duodenal aspirate pH decreased significantly after food intake (pH 6.8±0.2 vs. pH 6.0±0.1; p=0.01). In patients, no significant difference of pH was found between fasted and fed state (pH 6.9±0.3 vs. pH 6.5±0.1; p=0.16). HV displayed a lower pH than patients after meal intake (pH 6.0±0.1 vs. pH 6.5±0.1; p=0.002).

3.3.3 Patients with functional dyspepsia displayed decreased fasting bile acid concentrations

Total BA over time were similar in patients and HV (7.1±2.0mM vs. 9.1±2.0mM; Anova p=0.21; Figure 3.1A). However, total BA were decreased in patients compared to HV during fasting (1.8±0.3mM vs. 3.6±0.5mM; Anova p=0.03; Figure 3.1B). During fed state, total BA were similar in both groups (11.4±1.7mM vs. 13.2±2.1mM; Anova p=0.46; Figure 3.1C). BA peak concentrations occurred both in volunteers (18.8±2.7mM vs. 3.6±0.5mM; p<0.0001) and in patients (14.5±2.0mM vs. 1.8±0.3mM; p<0.0001) thirty minutes after liquid meal intake. These total BA peak concentrations were similar between patients and HV (p=0.3). TUDCA and GUDCA were present in small amounts in human intestinal fluid, while GCA and GCDCA were the most abundant BA. Unconjugated BA and LCA were present at or below the detection limit in aspirated intestinal fluids. The pooled concentrations of secondary BA (GDCA, TDCA, GUDCA and TUDCA) were similar in HV and patients (Figure 3.2B). On the other hand, the pooled concentrations of primary BA (GCA, TCA, GCDCA and TCDCA) were decreased in patients compared to HV during fasting (1.3±0.2mM vs. 2.9±0.4mM; Anova p=0.03; Figure 3.2A). Interestingly, the ratio of primary over secondary BA was lower in patients compared to HV during fed state (3.7±0.1 vs. 5.2±0.2; Anova p=0.04; Figure 3.2C). When we compared the individual BA between patients and controls, GCA, GCDCA and TCDCA concentrations were decreased in patients with FD during fasting. Also, GDCA percentages were increased and TCDCA percentages were decreased during fasting in patients with FD compared to HV. However, none of these differences of individual BA remained significant after correction for multiple testing. Health state but not gender significantly determined the variation in total BA during fasting (Anova p=0.03 with 12.9% of total variation and Anova p=0.1 with 5.7% of total variation, respectively). In addition, no correlation was found between age and BA concentration in both groups (p=0.5, spearman=-0.2 in FD; p=0.7, spearman=0.09 in HV). Finally, IBS comorbidity had no effect on BA concentrations in patients with FD and HV.

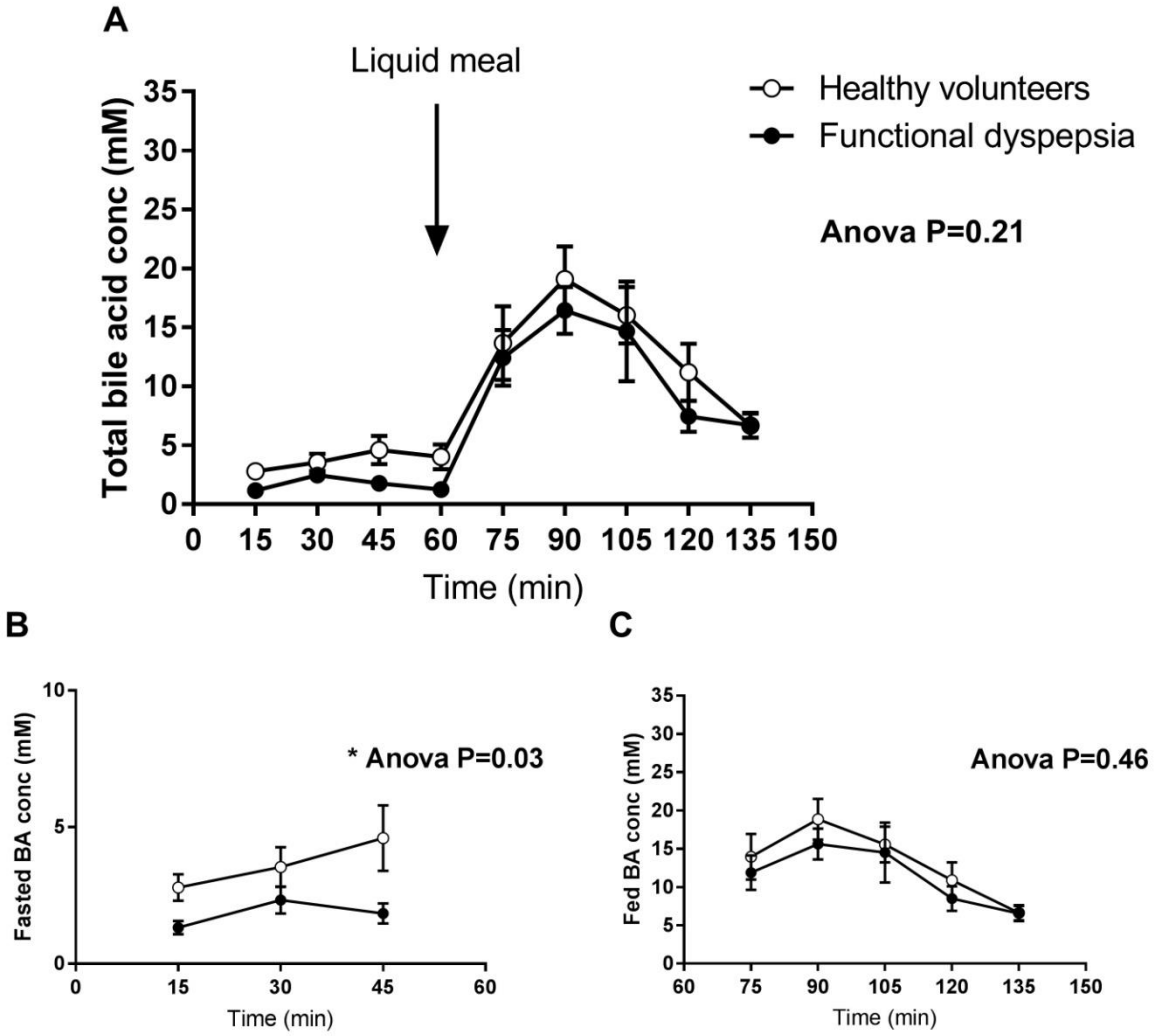


Figure 3.1 Total bile acid concentration in healthy volunteers and patients with functional dyspepsia. (A) This graph shows the total BA concentration per time point during 2.5 hours in HV and patients. During the first hour, the participants were fasted. During the last 1.5 hour, the participants were in fed state after an oral liquid meal intake. The BA concentrations in function of time were similar in patients and HV. (B) The average total BA concentration during fasting was lower in the patient group compared to the control group. (C) After food intake, the average total BA concentration was similar in both groups. The P-values were calculated with two-way ANOVA. BA, bile acid.

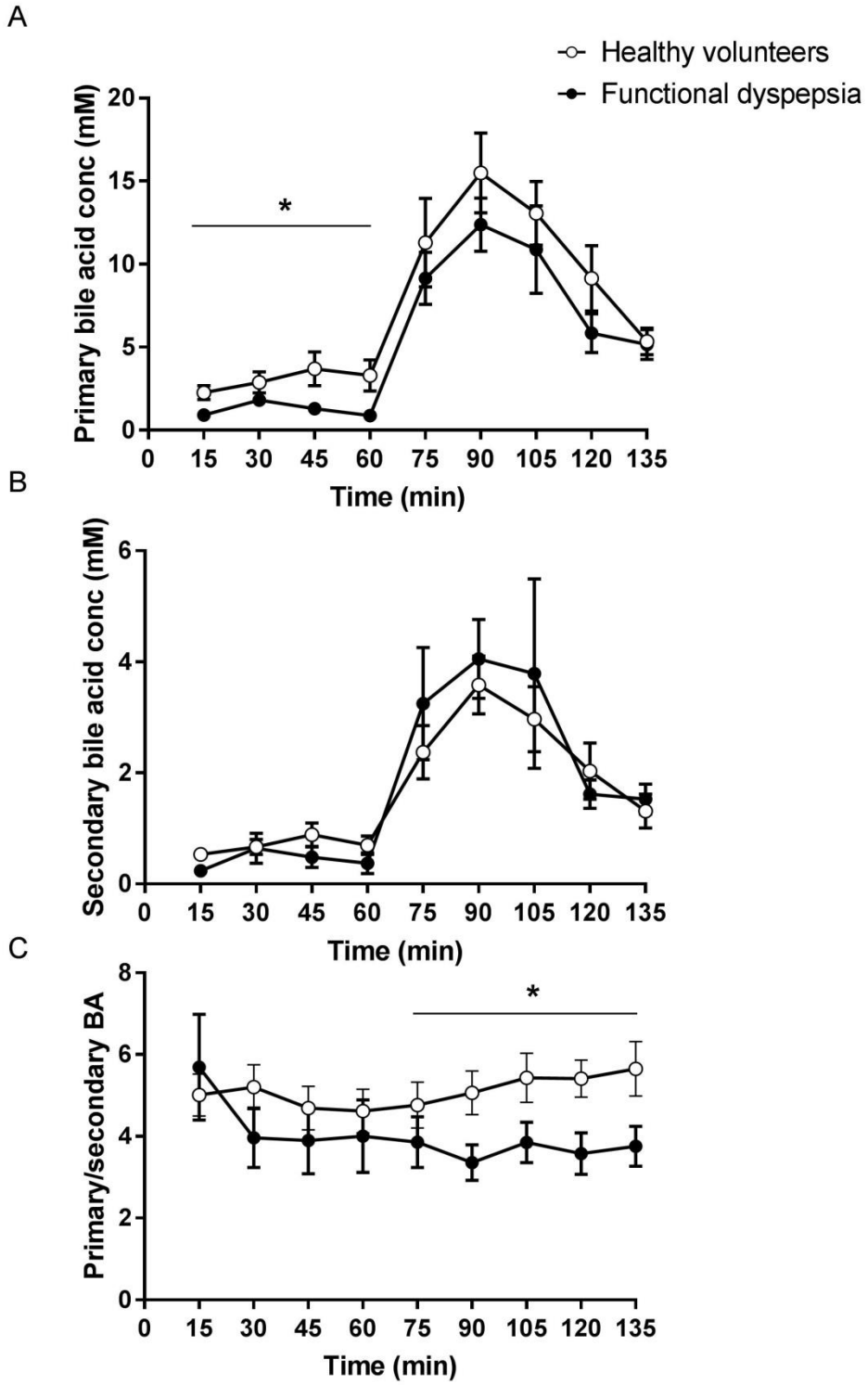


Figure 3.2 Primary and secondary bile acid concentrations in healthy volunteers and patients with functional dyspepsia. (A) The primary BA concentrations were lower in patients with FD compared to HV during fasting. (B) The secondary BA concentrations were similar in both groups. (C) The primary to secondary BA ratio was decreased in patients with FD compared to HV during fed state. The P-values were calculated with two-way ANOVA. BA, bile acid.

3.3.4 Patients with functional dyspepsia displayed duodenal vitamin D receptor upregulation

An increased duodenal VDR protein expression was shown in patients with FD compared to HV (3.7±1.0-fold; p<0.0005; figure 3.3A). Duodenal PXR and TGR5 expression did not differ significantly between patients and HV (0.8±0.2-fold; p=0.9 and 1.6±0.6-fold; p=0.4, respectively; Figure 3.3B&C). No or very low CAR and FXR protein expression could be detected in duodenal biopsies in both groups. Furthermore, health state and gender significantly determined the variation in VDR expression (p=0.0003 with 24.2% of total variation and p=0.02 with 8.6% of total variation, respectively); male (N=9) as well as female (N=15) patients with FD had higher expression compared to male (N=12) and female (N=12) HV (5.7±1.6-fold; p=0.004 and 2.5±0.8-fold; p=0.003, respectively).

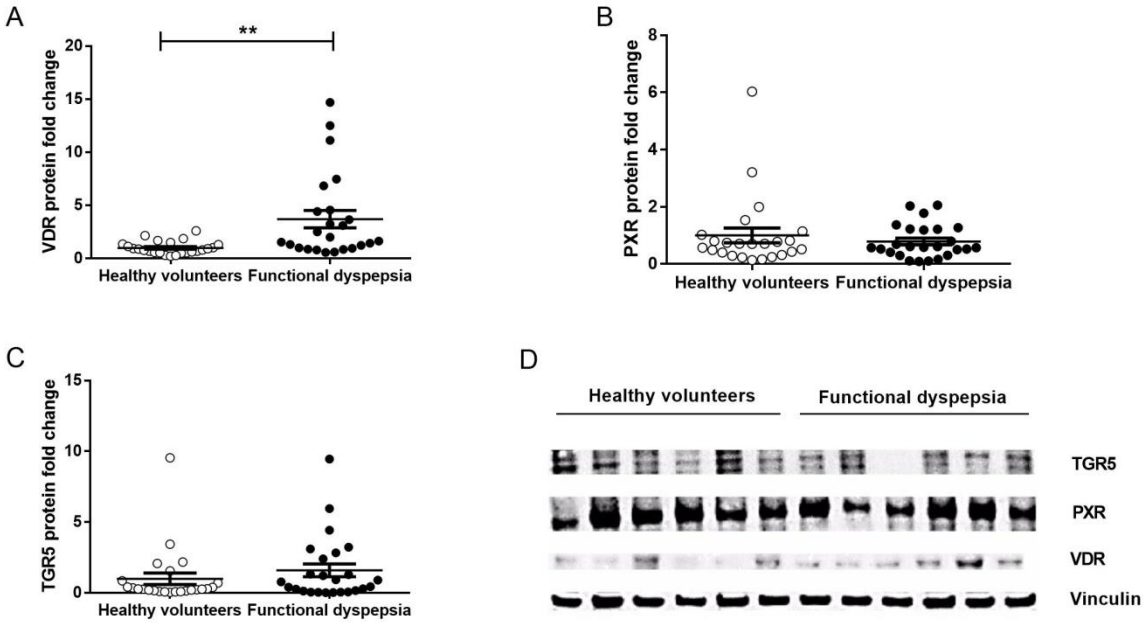


Figure 3.3 Expression of bile acid receptors in the duodenal mucosa. Duodenal TGR5, PXR, VDR and housekeeping gene vinculin protein expression was evaluated by western blot. Results were normalized to the housekeeping protein, vinculin, and protein fold change was determined relative to the mean value of the control group. (A) Patients with FD displayed an increased duodenal VDR protein expression compared to HV. (B&C) Duodenal PXR and TGR5 expression did not differ significantly between patients and HV. N=25 for both groups; **=p<0.01. (D) Representative western blot of six HV and six patients with FD. PXR, pregnane X receptor; TGR5, transmembrane G-protein-coupled receptor 5; VDR, vitamin D receptor.

3.3.5 Duodenal vitamin D receptor expression correlated with secondary bile acid concentrations in healthy volunteers

We evaluated the association between VDR expression and BA concentrations in fasted and fed state in patients and volunteers by correlation analysis (Table 3.3). A positive correlation was found between duodenal VDR expression and secondary BA concentration (Spearman=0.54; p=0.01) and a negative correlation was seen between VDR expression and the ratio of primary to secondary BA (Pearson=-0.55; p=0.01) after food intake in HV. When we pooled the results from both groups, a positive correlation was found between VDR expression and secondary BA concentration after food intake (Spearman=0.36; p=0.03). However, none of the correlations remained significant after correction for multiple testing.

Table 3.3 Correlation between duodenal VDR protein expression and bile acids

	Control group		**Patient group**		**All participants**	
Correlation with duodenal VDR protein expression	**P-value**	**correlation coefficient**	**P-value**	**correlation coefficient**	**P-value**	**correlation coefficient**
Fasted state						
Total bile acids	0.44	0.19	0.46	-0.19	0.14	-0.25
Primary bile acids	0.40	0.21	0.66	-0.11	0.19	-0.22
secondary bile acids	0.25	0.28	0.08	-0.44	0.32	-0.17
Primary/secondary bile acids	0.09	-0.40	0.53	0.17	0.97	-0.01
Fed state						
Total bile acids	0.64	0.11	0.79	0.08	0.96	-0.01
Primary bile acids	0.91	-0.03	0.45	0.21	0.60	-0.09
secondary bile acids	**0.01**	**0.54**	0.61	0.14	**0.03**	**0.36**
Primary/secondary bile acids	**0.01**	**-0.55**	0.46	0.21	0.51	-0.11

No correlation remained significant after Bonferroni correction for multiple testing. VDR, vitamin D receptor.

3.3.6 Bile acid concentrations correlated positively with epigastric burning

Several correlations were found between BA concentrations and dyspeptic symptoms (Table 3.4). With correction for multiple testing, the positive correlation between epigastric burning and total BA concentrations in patients during fasting and fed state remained significant.

Table 3.4 Correlation between clinical factors and total bile acids

	Patients with functional dyspepsia			
	Fasted state		**Fed state**	
Clinical factors	**P-value**	**Correlation coefficient**	**P-value**	**Correlation coefficient**
VSI	0.09	0.43	0.27	0.30
PHQ-9	0.19	0.34	**0.03**	**0.56**
DSS	**0.004**	**0.66**	0.21	0.34
Weight loss	0.14	0.38	0.77	0.08
Discomfort	0.31	0.26	0.49	0.19
Postprandial fullness	0.64	0.12	0.29	-0.29
bloating	0.07	0.45	0.49	0.19
Epigastric pain	**0.01**	**0.59**	0.08	0.47
Early satiation	0.30	-0.26	0.33	-0.27
Nausea	0.10	0.41	0.13	0.41
Vomiting	0.71	0.15	0.40	-0.31
Belching	0.09	0.43	0.89	0.04
Epigastric burning	**<0.0001**	**0.83**	**0.0004**	**0.80**
Sternal burning	**0.03**	**0.53**	0.43	0.23

Only the symptom epigastric burning remained significant after correction for multiple testing. N=15 patients.
VSI, visceral sensitivity index; PHQ-9, patient health questionnaire 9; DSS, dyspepsia symptom score.

3.4 Discussion

In the last few years, there is an increasing interest in duodenal alterations in patients with FD.[2-4, 14] In the present paper, we report decreased total duodenal BA concentrations during fasting and increased duodenal VDR expression in FD compared to controls.

The analysis of duodenal fluids showed a similar fasting pH in patients with FD and HV. After meal intake, however, the pH of duodenal aspirates from HV decreased, while the pH of duodenal aspirates from patients remained similar to fasting. In line with our results in HV, Wuyts et al. reported a pH of duodenal aspirates from HV in fasted and fed state of 7.5 and 6.3, respectively.[15] The higher postprandial pH in patients with FD is in apparent conflict with the results from a previous study by Lee et al., in which reduced duodenal acid clearance and increased late postprandial duodenal acid exposure was reported in a selected group of dyspeptic patients with prominent nausea.[16] The different results may be related to differences in patient selection, meal choice, method of pH measurement and especially study design, with a shorter postprandial measurement time in the present study compared to the 24h duodenal pH recording in the study by Lee et al, where differences occurred mainly after the second postprandial hour.

Our main finding was decreased total BA concentration during fasting in patients with FD. According to the literature, dietary components and intestinal inflammation can provoke changes in the intestinal microbiome and the microbiome can lead to changes in the BA pool.[8, 17] BA on the other hand can influence the intestinal microbiome by their antibacterial properties and inhibit the intestinal bacterial endotoxin absorption by their anti-inflammatory properties.[8, 17-18] Decreased BA levels are linked to increased bacterial load and inflammation.[19] In view of this, our results suggest that decreased BA levels, may contribute to duodenal changes such as increased permeability and (low-grade) inflammation, possibly through increased translocation of bacterial products.[3] On the other hand, our statistically significant data do not necessarily indicate that this decreased duodenal BA concentration has a significant biological effect in patients with FD. Further research is necessary to establish to which extent an altered luminal BA composition plays a role in the pathophysiology of FD. Treatment trials with administration of exogenous BA (ursodeoxycholic acid, obeticholic acid) may be required to fully address this issue.

In the present study, we demonstrated decreased primary BA concentrations and a shifted primary to secondary BA ratio in patients with FD. Primary BA are more hydrophilic than secondary BA. Hydrophilic BA have cytoprotective effects, whereas hydrophobic BA have cytotoxic effects.[8] Therefore, the hydrophobic nature of the BA pool is linked with pathological conditions.[19] This finding

of a decreased primary to secondary BA ratio in patients with FD may contribute to impaired duodenal barrier function.[3, 14] In addition, we report no effect of gender and age on BA levels. Conflicting data have been reported on male and female plasma BA concentrations.[20] However, our data concerns luminal BA concentrations.

Furthermore, duodenal VDR expression is almost 4-fold increased in our patient group. VDR regulates BA metabolism and synthesis and is involved in the action of secondary BA.[8, 18] VDR is also involved in cell differentiation, inhibition of proliferation and immunoregulation.[21] Kong et al. demonstrated that VDR can protect the gastrointestinal tract from BA toxicity and preserve mucosal barrier homeostasis.[21] Vitamin D3 is a strong VDR ligand which inhibits proinflammatory cytokine production, alters the gastrointestinal microbiome and increases bacterial diversity.[8,22-24] These actions of vitamin D explain its positive interference in inflammatory disorders.[25] It is conceivable that the upregulation of VDR in FD is part of a mucosal defense against low-grade inflammation, as has been observed in other epithelia.[26-27] However, there are two alternate explanations for duodenal VDR upregulation in FD. First, VDR expression shows great variability in the duodenum.[28] Inconsistent location of biopsy sites may thus induce variability. However, biopsy preservation was performed from approximately the same position in the second part of the duodenum, indicating that this is unlikely to underlie a significantly increased VDR expression in FD compared to HV. Second, VDR upregulation may be due to a vitamin D deficiency present in the patient group. In view of this, evaluation of the vitamin D status of patients with FD is important, but was beyond the scope of this study.

Although there is no change in TGR5 and PXR expression, an altered BA composition may lead to an altered activation. TGR5 recognizes TDCA, TCDCA, TCA and taurolitocholic acid (TLCA) and plays a role in prevention of inflammation.[9] Our results show that TCDCA is present in lower levels in patients during fasting. This may suggest that TGR5 is activated to a lesser extent in patients with FD, which may add to the induction/maintenance of duodenal low-grade inflammation. On the other hand, PXR has a role in elimination and detoxification of LCA, which strongly binds to PXR.[9] However, LCA is not detectable in duodenal fluid. So, we can't imply whether PXR plays a role in this pathology. Moreover, we demonstrated that being male and having FD is associated with an increased duodenal VDR expression.

Apart from vitamin D, gut microbiome and secondary BA can also regulate VDR.[29] Duodenal VDR expression correlates positively with the sum of the secondary BA concentrations in HV. The correlation suggests that an increase in secondary BA levels may induce duodenal VDR upregulation.

However, this correlation is not present in patients, where the VDR is already upregulated, potentially as a mucosal protective measure. The pathophysiological relevance of altered duodenal BA to the symptom pattern in FD needs more investigation as we found several correlations, including a positive correlation between BA and epigastric burning. It is conceivable that the altered BA concentration has indirect effects on symptom generation, for instance through altered mucosal integrity, low-grade inflammation and a change in feedback control of gastric emptying and accommodation.[2]

The strengths of this study are the heterogeneous group of patients representing FD without focusing on a certain subtype, and the use of LC-MS/MS for absolute quantitation of luminal BA. It should be pointed out that the volunteers and patients are not gender- and age-matched, resulting in an older patient group. However, we found no evidence of age- and gender-related changes in bile aspirate composition. In addition, it remains uncertain whether these changes in total BA and VDR expression are a cause of the pathophysiological mechanisms in FD or reflect a consequence of the change in diet in patients who often suffer from postprandial dyspeptic symptoms. Hence, a causal relationship needs to be established in future experiments. Finally, vitamin D blood levels and the marker for BA synthesis need to be measured in patients in future studies in order to validate the present results.

Overall, we report a decreased duodenal BA concentration during fasting and an increased duodenal VDR expression in FD. This may reveal a tip of a complex interaction between BA, microbiome and VDR underlying the pathophysiological mechanisms in FD. However, further research is needed to fully understand the role of BA in the pathophysiology of FD.

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Chapter 4

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

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

4.1 Introduction

Patients with 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 duodenal mucosal low-grade immune activation and impaired duodenal barrier function.[3]

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 BA.[3,4-12]

BA inhibit bacterial proliferation and have antimicrobial and amphipathic properties allowing them to influence the gut microbiome.[13] High concentrations of hydrophobic secondary BA can lead to DNA damage and cell death.[13] Moreover, hydrophobic BA were shown to impair the small intestinal barrier function and increase the intestinal permeability.[14-17]An impaired intestinal barrier function may in turn result in BT, which is the passage of bacteria and bacterial products across the intestinal barrier, as reported for colonic biopsies of IBS patients.[18,19, 20] In a study by Münch et al, it was shown that hydrophobic BA worsen mucosal barrier dysfunction in colonic biopsies of patients with collagenous colitis in remission, resulting in increased bacterial uptake.[21]

A decreased duodenal BA concentration was recently discovered by our group in patients with FD.[22] For this purpose, we hypothesized that these alterations in the duodenal BA concentration in FD may contribute to the impaired duodenal mucosal integrity, resulting in increased BT. Therefore, we aimed to evaluate the relationship between BA, BT and duodenal mucosal permeability in patients with FD and HV.

4.2 Materials and methods

4.2.1 Study population

Patients fulfilling Rome IV criteria for FD, who were scheduled for upper gastrointestinal (GI) endoscopy with duodenal biopsies, were prospectively recruited at the outpatient clinic of the Department of Gastroenterology at the University Hospitals Leuven.[3] 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.[3] None of the participants was allowed to take antihistamines, ketotifen, cromoglycate, acetylsalicylates, non-steroidal anti-inflammatory drugs, anticholinergics, theophylline, β2-agonists, codeine or opioid derivatives and proton pump inhibitors for at least 2 weeks prior to the gastroscopy.[3] Psychosocial co-morbidity was assessed by the PHQ-9 and the VSI questionnaire was used to evaluate gastrointestinal-specific anxiety. The DSS was used to score the dyspeptic symptom intensity over the preceding 3 months in patients with FD and HV.[23] 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.

4.2.2 Duodenal mucosal integrity, fluid aspiration and bile acid analysis (s56910)

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 1.76mm^2, following the same protocol as stated by Vanheel et al.[3] Mucosal integrity was measured as TEER and paracellular passage of a 4kDa fluorescein isothiacyanate-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 (Fortimel Energy®; 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 BA concentration was analyzed with LC-MS/MS, as previously reported.[22, 24] BA were expressed as absolute concentrations (mM) and total BA was the molar sum of all measured BA.[22] Fasting and fed BA were calculated as the average of all time points in fasted and fed state.[22] Primary BA were the molar sum of GCA, TCA, GCDCA and TCDCA. Secondary BA were the molar sum of GDCA, TDCA, GUDCA and TUDCA. The BA peak occured approximately 30 minutes after liquid meal intake and was characterized by the maximal amount of total BA postprandially.

4.2.3 Evaluation of bacterial uptake (s56880)

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 1.76mm^2 using the technique previously described by Wallon et al.[25] 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 carbogenated 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, 21] TEER was measured for 120 min in all biopsies.[21] 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 2.10^6 *E. Coli* units/ml.[26] Bacteria were killed with paraformaldehyde which stops their reproduction but retains antigenicity.[26] 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.[21] We analyzed the data concerning the separate (1, 2 and 5mM) and the pooled GUDCA concentrations (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 Labech, Ortenberg, Germany) at 480nm excitation and 520nm emission level.[26] We calculated the *E. Coli* units from fluorescence intensity using an *E. Coli* standard curve. In addition, 2 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.[21] 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. This method was taught by the Department of Clinical and Experimental Medicine at Linköping University (Linköping, Sweden) and applied in TARGID at KU Leuven (Leuven, Belgium).

4.2.4 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).

4.3 Results

4.3.1 Study population (s56910)

Three duodenal biopsies and aspirates were obtained from 18 patients with FD (35.4±3.3 years, 6 men, Table 4.1). Table 4.2 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.[27]

Table 4.1 Demographics and clinical characteristics of the participants

Characteristic	Patients with functional dyspepsia
Gender, Female:Male	12:6 (66.7% female)
Age, years (range)	35.4±3.3 (18-57)
Dyspeptic symptoms, DSS	10.3±1.1
BMI, kg/m²	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 4.2 Frequency of severity grading by DSS for each of the nine dyspeptic symptoms in patients with functional dyspepsia

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.

4.3.2 Correlation between bile acids and duodenal permeability in patients with functional dyspepsia

This patient group showed an average TEER of 20.7±1.2Ω.cm^2 and permeability to FITC-dx4 of 35.8±4.1pmol. TEER and paracellular passage were negatively correlated ($r=-0.82$; $p<0.0001$).

We evaluated possible correlations between individual BA, total BA, primary BA and secondary BA concentrations and paracellular passage and TEER (Table 4.3). 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; Figure 4.1A) and the fed state ($r=0.61$; $p=0.02$; N=14; Figure 4.1B). In addition, a positive correlation was found between secondary BA and paracellular passage ($r=0.60$; $p=0.03$, N=13; Figure 4.1C) during the BA peak. However, no correlations survived Bonferroni correction for multiple testing.

Table 4.3 Correlation analysis between bile acid concentration and duodenal permeability in FD						
Correlation with duodenal permeability	**TEER**			**Paracellular passage**		
	P-value	**correlation coefficient**	**N**	**P-value**	**correlation coefficient**	**N**
Fasted state						
Glycocholic acid	0.78	0.07	17	0.89	0.04	15
Glycochenodeoxycholic acid	0.87	-0.04	17	0.99	-0.005	15
Glycodeoxycholic acid	0.34	0.25	17	0.55	-0.17	15
Glycoursodeoxycholic acid	0.66	-0.12	17	0.57	-0.16	15
Taurocholic acid	0.97	-0.01	17	0.61	0.14	15
Taurochenodeoxycholic acid	0.72	0.09	17	0.83	0.06	15
Taurodeoxycholic acid	0.46	0.19	17	0.70	-0.11	15
Tauroursodeoxycholic acid	0.40	-0.22	17	0.59	0.15	15
Primary bile acids	0.88	0.04	17	0.94	0.02	15
Secondary bile acids	0.79	0.07	17	0.86	-0.05	15
Total bile acids	0.76	0.08	17	0.73	0.10	15
GCA+GCDCA/GUDCA+TUDCA	**0.03**	**0.56**	**15**	0.57	-0.17	13
Fed state						
Glycocholic acid	0.97	-0.01	15	0.22	0.36	13
Glycochenodeoxycholic acid	0.52	-0.18	15	0.51	0.20	13
Glycodeoxycholic acid	0.52	0.18	15	0.33	0.30	13
Glycoursodeoxycholic acid	0.19	-0.36	15	0.97	0.01	13
Taurocholic acid	0.39	0.24	15	0.66	0.14	13
Taurochenodeoxycholic acid	0.97	-0.01	15	0.67	0.13	13
Taurodeoxycholic acid	0.81	0.07	15	0.52	0.20	13
Tauroursodeoxycholic acid	0.15	-0.39	15	0.33	0.30	13
Primary bile acids	0.48	-0.20	15	0.28	0.32	13
Secondary bile acids	0.93	0.03	15	0.08	0.50	13
Total bile acids	0.92	0.03	15	0.09	0.49	13
GCA+GCDCA/GUDCA+TUDCA	**0.02**	**0.61**	**14**	0.85	-0.06	12
Bile acid peak						
Glycocholic acid	0.80	-0.07	15	0.47	0.22	13
Glycochenodeoxycholic acid	0.97	-0.01	15	0.94	-0.03	13
Glycodeoxycholic acid	0.89	-0.04	15	0.14	0.44	13
Glycoursodeoxycholic acid	0.67	-0.12	15	0.59	-0.17	13
Taurocholic acid	0.94	-0.02	15	0.62	0.15	13
Taurochenodeoxycholic acid	0.77	0.08	15	0.70	0.12	13
Taurodeoxycholic acid	0.98	-0.009	15	0.37	0.27	13
Tauroursodeoxycholic acid	0.31	-0.28	15	0.38	0.26	13
Primary bile acids	0.57	-0.16	15	0.38	0.27	13
Secondary bile acids	0.60	-0.15	15	**0.03**	**0.60**	**13**
Total bile acids	0.44	-0.21	15	0.15	0.42	13

No correlations remained significant after correction for multiple testing. FD, functional dyspepsia; TEER, transepithelial electrical resistance; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GUDCA, glycoursodeoxycholic acid; TUDCA, tauroursodeoxycholic acid.

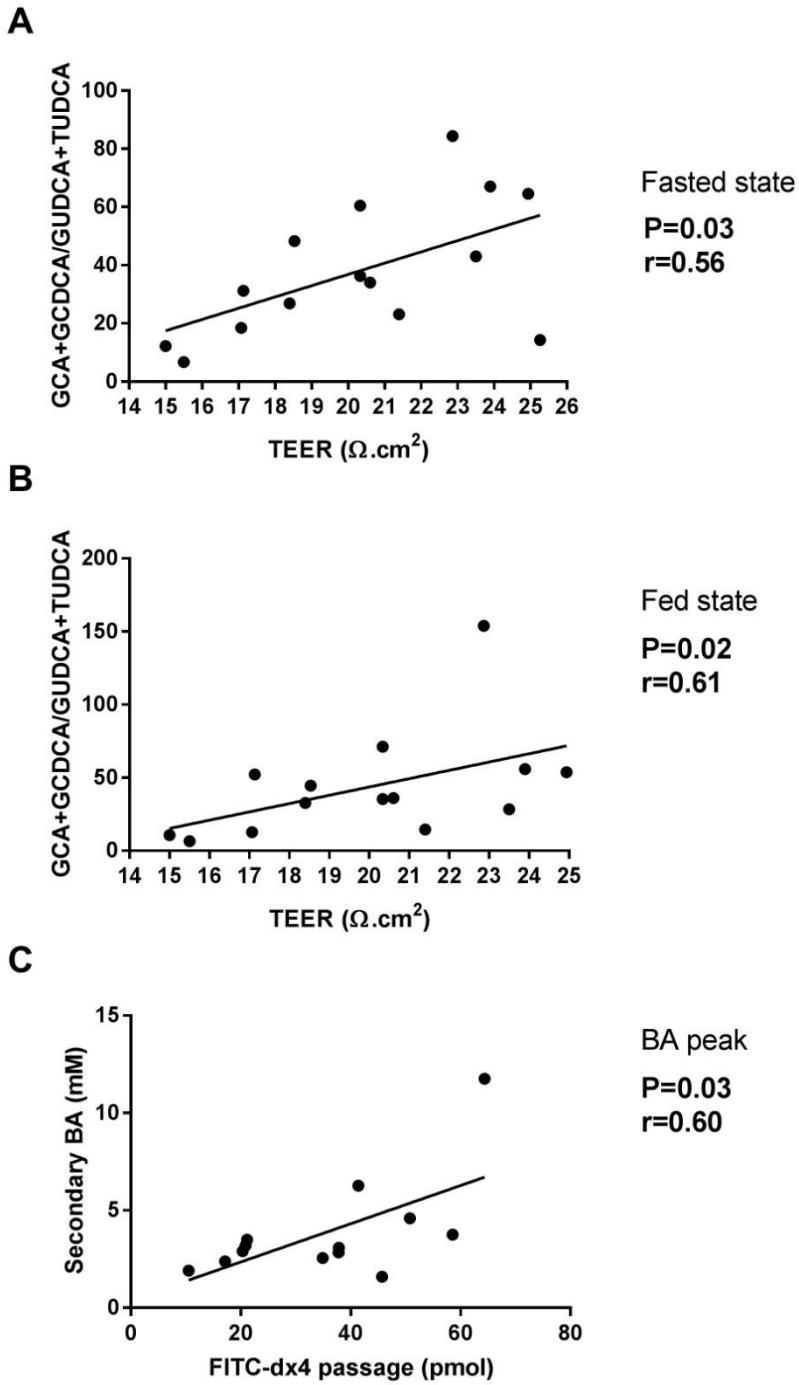


Figure 4.1 Correlation between bile acid and duodenal permeability in patients with functional dyspepsia. (A-B) A positive correlation was found between the ratio of GCA and GCDCA to TUDCA and GUDCA and TEER in fasted and fed state. (C) A positive correlation was found between secondary BA and paracellular passage during the BA peak. However, no correlations survived Bonferroni correction for multiple testing. BA, bile acids; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; GUDCA, glycoursodeoxycholic acid; TEER, transepithelial electrical resistance.

4.3.3 Study population (s56680)

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 4.4). Table 4.5 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.[27]

Table 4.4 Demographics and clinical characteristics of the participants

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/m²	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 4.5 Frequency of severity grading by DSS for each of the nine dyspeptic symptoms in patients with functional dyspepsia

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.

4.3.4 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±1.3Ω.cm^2 vs., p=0.02, N=21 for each group; Figure 4.2A). 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 4.2B).

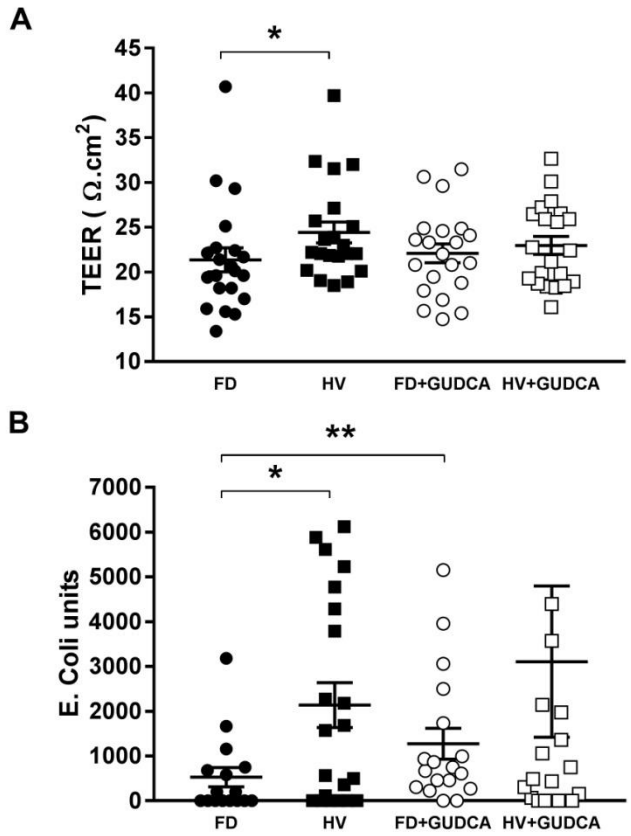


Figure 4.2 Glycoursodeoxycholic acid increased the mucosal epithelial resistance of and the *E. Coli* passage in duodenal biopsies of patients with functional dyspepsia. We pooled the different concentrations of GUDCA. (A) The duodenal mucosal TEER was significantly lower in biopsies from patients with 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±SEM; *p<0.05, **p<0.01. GUDCA, glycoursodeoxycholic acid; FD, functional dyspepsia; TEER, transepithelial electrical resistance; HV, healthy volunteers.

4.3.5 Glycoursodeoxycholic acid abolished the differences in mucosal 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±1.0Ω.cm^2 vs. 21.4±1.3Ω.cm^2, p=0.5 and 23.0±1.0Ω.cm^2 vs. 24.4±1.2Ω.cm^2, p=0.1, respectively; Figure 4.2A). 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 4.2B). 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 (Figure 4.3).

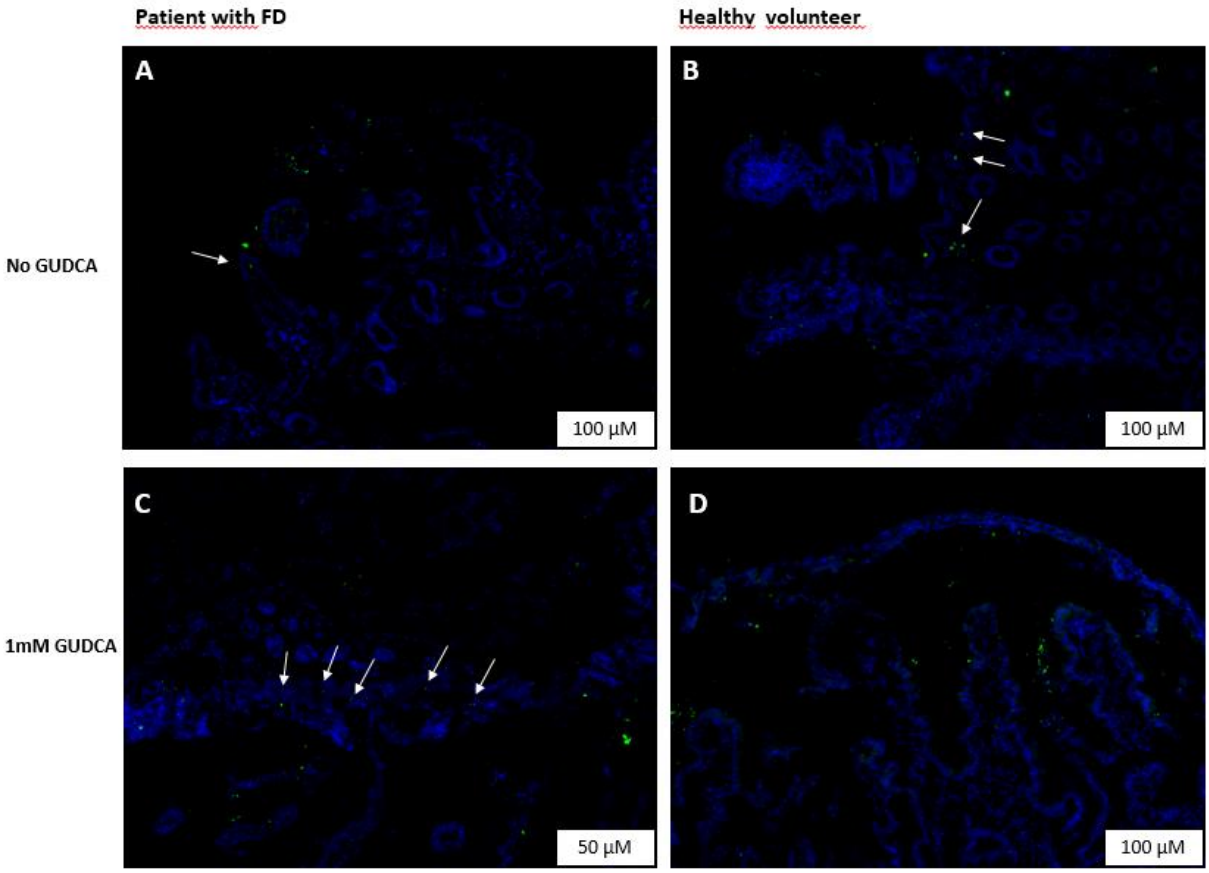


Figure 4.3 Fluorescent *E. Coli* were present in the epithelial cell layer of patient and healthy volunteer biopsies. Fluorescent *E. Coli* (green and indicated with arrows) were present in the epithelial cell layer of (A) a patient and (B) a HV biopsy without GUDCA exposure and of a (C) patient and (D) a HV biopsy exposed to 1mM GUDCA for 120 minutes. FD, functional dyspepsia; GUDCA, glycoursodeoxycholic acid.

4.3.6 Exposure to 1mM glycoursodeoxycholic 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 (Figure 4.4A&C). GUDCA also showed no concentration-dependent effect on TEER in HV biopsies (Figure 4.4B). 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 4.4D).

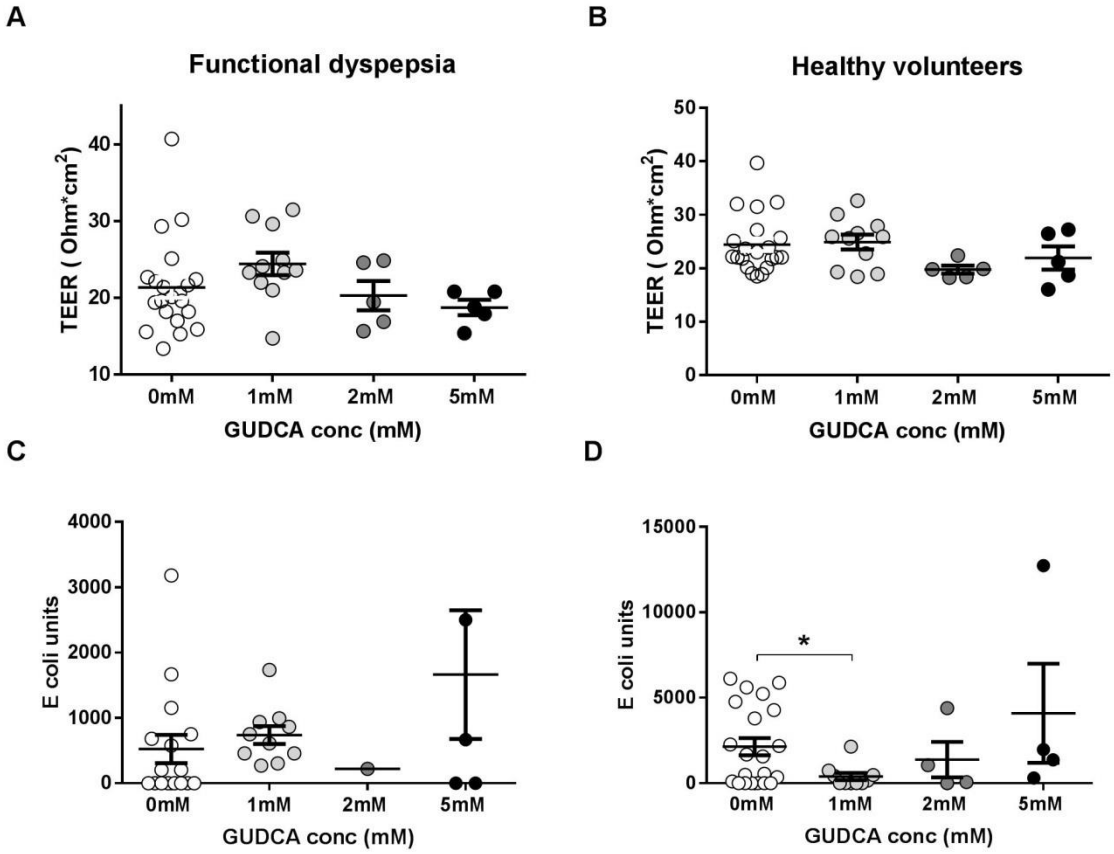


Figure 4.4 Exposure to 1mM glycoursodeoxycholic acid decreased *E. Coli* passage in duodenal biopsies of healthy volunteers. Patient and HV duodenal biopsies were exposed to 0mM GUDCA (N=21), 1mM GUDCA (N=11), 2mM GUDCA (N=5) or 5mM GUDCA (N=5). (A-B) No effect of GUDCA on TEER in patient and HV biopsies. (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±SEM; *p<0.05. GUDCA, glycoursodeoxycholic acid; TEER, transepithelial electrical resistance.

4.3.7 No correlation between duodenal permeability and bacterial passage

After correlation analysis, only a strong correlation was found 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.

4.4 Discussion

In this study, we aimed to unravel the relationship between BA, BT and duodenal mucosal permeability in patients with FD and HV. First, we observed a complex correlation between BA and duodenal permeability in patients with FD, in which the hydrophilic BA 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 between secondary BA and paracellular passage during the BA peak in patients with FD. In agreement with our hypothesis, these data confirmed a correlation between BA and duodenal permeability in patients with FD. Both correlations link increased GUDCA and TUDCA concentrations to increased duodenal permeability in patients with FD. However, these correlations did not survive correction for multiple testing, and 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.[28] However, the data from the literature are based on the effect of pharmacological concentrations of exogenous UDCA rather than concentrations of the endogenous BA. We tested this particular ratio of GCA and GCDCA to TUDCA and GUDCA based on the alterations seen in the BA concentrations of patients with FD compared to HV in chapter 3.[22]

UDCA is an hydrophilic secondary BA with anti-apoptotic and anti-inflammatory effects that may have therapeutic effects on the colonic intestinal barrier in inflammatory bowel disease (IBD).[28-34] In addition, UDCA increases the hydrophilicity index of the circulating BA pool, stimulates bile secretion and protects against BA and cytokine toxicity.[35] It has already been described that intestinal microbiota, food and bile have a significant role in indomethacin-induced small intestinal inflammation and barrier dysfunction in rats.[36] 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.[34, 37] 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.[36] Oral intake of TCDCA, on the other hand, attenuated intestinal inflammation and prevented indomethacin-induced increases in the hydrophobicity index.[36]

Our results and the reported effects of UDCA, UDCA conjugates and other BA on intestinal permeability and inflammation may differ due to the use of cancer 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.[28] Moreover, 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.[28] Considering the migratory and anti-inflammatory effects, UDCA may be useful in the treatment of disorders involving small intestinal damage.[28]

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 this 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 is in agreement with the existing literature, showing that UDCA may stabilize membranes.[38] Esteves et al investigated the effects of the hydrophobic BA DCA and hydrophilic BA UDCA on monolayers and liposomes.[38] They showed that DCA caused fluidization and rupture of monolayers and that UDCA may stabilize membranes.[38]

Furthermore, contrary to our hypothesis, less bacterial passage was observed in biopsies from patients with FD compared to HV and 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. According to the literature, BT occurs less frequently in the small intestine compared to the large intestine.[38-39] In a study by Münch et al, exposure of colonic biopsies from patients with collagenous colitis and HV to 100 µmol⁄L CDCA or DCA in Ussing chambers resulted in an increased *E. coli* K12 uptake in biopsies from patients with collagenous colitis in remission.[25] 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.[25] In the present study, we studied the effect of 1, 2 and 5mM of UDCA, a BA known for its cytoprotective effect, on duodenal – rather than colonic – biopsies in a different disorder, which may all account for the different conclusions.

UDCA is available as a therapeutic agent and part of the goal of the study was to explore a potential mechanism of UDCA in patients with FD. It was shown that BA modulate barrier function via EGFR or reactive oxygen species in Caco-2 cells and that nonpathogenic *E. coli* strains ameliorate barrier

function.[29, 31, 40-41] Münch et al hypothesized that luminal BA, in susceptible hosts, may induce colonic inflammation due to unknown mechanisms resulting in hypersensitivity to BA.[25] 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.[42] Esteves et al suggested that the exposure of monolayers to a combination of DCA and UDCA had a greater protective effect than individual BA.[38] Based on the literature and on our own results, we suggest that UDCA and its conjugates work most efficiently *in vivo* when in combination with other BA and with microbial metabolization of UDCA into LCA for its fully protective effect. In the present *in vitro* study, exposure of duodenal tissue to high concentrations of the cytoprotective BA, 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 does not exceed the levels of biopsies from HV. The protective effect of GUDCA in this case is only present in the form of a normalized TEER and is probably lacking due to exposure to high concentrations.

Duodenal biopsies were exposed to 1, 2 or 5mM GUDCA for 2 hours. Only 1mM GUDCA decreased bacterial passage through HV biopsies. However, we have to be careful, as the sample size of the 2 and 5mM group is small. Furthermore, these concentrations are quite 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±0.02mM.[22]

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 BT is probably not altered in patients with FD and no part of the pathophysiological mechanisms of FD, as far as we are able to evaluate using a duodenal and an FD unspecific, chemically killed bacterium (*E. Coli*). It remains to be shown whether indeed BT is absent in lower parts of the GI tract with higher bacterial exposure. Moreover, our analysis was limited to a single inactivated strain of bacteria (*E. Coli*) which may not be relevant in the pathogenesis of FD. Based on the emerging data, duodenal BT and other effects of disease-specific strain can be investigated. Indeed, in a small study from Zhong et al, 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; duodenal mucosal bacterial load correlated negatively with quality of life; more severe symptom responses to the standardized

meal correlated positively with mucosal bacterial load; and finally, the total bacterial load correlated negatively with bacterial diversity.[43] Furthermore, the microbiome alters BA pool composition, which alters the BA pool size.[44] Gut bacteria can perform multiple biotransformations of BA, including the hydrolysis of conjugated BA by BSH, the 7α-dehydroxylation of CA into DCA and CDCA into LCA, 7β-dehydroxylation of UDCA into LCA and oxidation and epimerization (of OH-groups at position C3, C7 and C12) resulting in isobile acids.[45-46]

This *in vitro* 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 BA, one measure for permeability and one strain of bacteria, which is not specific for the duodenum, nor for FD and is chemically killed. Also, our data were analyzed by pooling the data from the different GUDCA concentrations.

In summary, the reported effects of GUDCA on duodenal permeability and *E. Coli* passage ask for more intensive research in the effects of individual as well as combined BA 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 BA in this experimental setup, but also living, duodenum and/or FD specific bacteria.

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Chapter 5

Effect of ursodeoxycholic acid on duodenal permeability, low-grade inflammation, gut microbiome and meal related sensory motor function in functional dyspepsia

5 Effect of ursodeoxycholic acid on duodenal permeability, low-grade inflammation, gut microbiome and meal related sensory motor function in functional dyspepsia

5.1 Introduction

FD is a heterogeneous disorder of the upper GI tract, defined by the Rome IV criteria as the presence of symptoms, including postprandial fullness, early satiation, epigastric pain and epigastric burning, originating from the gastroduodenal region without evidence of a structural disease at routine investigations.[1] Although FD is considered a functional disorder, our group has recently reported impaired duodenal barrier function in patients with FD at the level of the junction proteins.[2] This may result in the previously observed low-grade inflammatory changes in the duodenal mucosa, generating dyspeptic symptoms by inducing altered motor control and visceral hypersensitivity.[3] The cause of impaired duodenal barrier function is still unknown, but identifying the underying pathophysiology may lead to efficient and target-oriented treatment options for patients with FD.[4]

The endogenous balance of BA has become of great interest in FD research.[5] BA have already been recognized as crucial components in the pathogenesis of other functional GI disorders, including IBS, as their binding capacity to specific receptors mediates physiological and pathophysiological effects.[5] Moreover, the hydrophilic BA, UDCA, is considered a protective BA, because it can shield the epithelial membranes from the detergent effects of the hydrophobic BA.[6] Hence, the effect of UDCA on the intestinal mucosa of patients with FD is a relevant research target.[5]

In view of this, we aimed to investigate the potential protective effect of UDCA in patients with FD *in vitro* and *in vivo*.

5.2 Materials and methods

5.2.1 Effect of ursodeoxycholic acid on mucosal integrity *in vitro*

5.2.1.1 Caco-2 cell culture

Caco-2 cells (ATCC® LGC Standards S.a.r.l., France), were grown in cell culture medium (Dulbecco's modified Eagle's medium (DMEM, 85,9% with 4,5 g/L D-glucose, without L-glutamine, Gibco, Thermo-Fisher Scientific, Waltham, Massachusetts, USA), fetal bovine serum (FBS, VWR International, Leuven, Belgium), 1% penicillin/streptomycin (1000 IU/ ml/ 10000 µg/ ml, Lonza, Verviers, Belgium), 1% 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES, 1M, Lonza) and 2% nonessential amino acid (NEAA, 10 mM, Lonza)) at 37°C in an atmosphere of 5% CO_2 and 90% humidity.[5] When the cells were confluent, they were trypsinized, counted with a Bürker-Türk counting chamber and seeded onto polyester membranes on transwells (pore diameter of 0.4 µm, insert diameter of 12 mm and a growth area of 1.12 cm^2; Costar, Corning, New York, USA) at a density of 9.10^4 cells/cm^2.[5] The transwells were incubated at 37°C. The TEER of the Caco-2 cell monolayers was weekly measured with an EVOM epithelial volt-ohmmeter and STX2 electrode (World Precision Instruments, Sarasota, USA).[5] Transwells with a TEER of 500-600 Ω.cm^2 were ready for use.

5.2.1.2 Fasted state human intestinal fluid

Duodenal fluid aspirates from patients with FD and HV during fasted state were used to make two pools, one from the duodenal aspirates of patients with FD (FaHIFpool FD) and one from the duodenal aspirates of HV (FaHIFpool HV).[5] Both pools were characterized by LC-MS/MS and the pH of both pools was adjusted to pH 6,5 by using 2-N-morpholinoethanesulfonic acid (MES, Sigma-Aldrich, St. Louis, Missouri, USA).[5]

5.2.1.3 Transwell experiment

The apical compartments of the transwells were washed thoroughly with HBSS+ (Hanks' balanced salt solution (HBSS, Gibco-Thermo Fisher Scientific, Watham, Massachusetts, USA), 25mM D(+)-glucose (MW 180,16 g/mol, Merck KGaA, Darmstadt, Germany) and 10mM HEPES; pH 7.4) and placed for 30 min at 37°C.[5] HBSS+ (pH 7.4) was removed from the apical compartments and 250 µl mucus (20 mg/ml type III porcine mucin, Sigma-Aldrich, St. Louis, Missouri, USA; pH 6.5) was added.[5] HBSS+ (pH 6.5) was added to the basolateral compartments, the transwell plates were incubated for 10 min at 37°C and TEER was measured.[5] Then, HBSS+ (pH 6.5) was removed – without removing the mucus layer – from the apical compartments and 300 µl of UDCA (20mM, MW 392.57 g/mol, Sigma-Aldrich, St. Louis, Missouri, USA), FaHIFpool FD and/or FaHIFpool HV was added to the apical

compartment.[5] Finally, TEER was measured after 0min, 30 min, 1, 2 and 3 hours. This experiment was performed twice with each 22 different conditions.

5.2.1.4 Cell viability

Caco-2 cells were grown in a 96 well plate (Tissue Culture Testplate 96U, Switzerland) and kept at 37°C.[5] Cells were exposed to each condition for 1 hour. Next, the fluid was removed and Cell Proliferation Reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) was added.[5] After an incubation period of 30 minutes at 37°C, the plate was shaken and analyzed with the fluorimeter (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) at 0, 30 and 60 minutes and at wavelength 440, 470 and 600nm.[5]

5.2.2 Effect of ursodeoxycholic acid on duodenal permeability, low-grade inflammation, gut microbiome and meal related sensory motor function in patients with functional dyspepsia

5.2.2.1 Study population

Ambulatory patients between 18 and 60 years, meeting the Rome IV criteria for FD were recruited at the gastroenterology outpatient clinic. Patients were excluded if they had a history of ulcerative colitis, Crohn's disease, lactose intolerance, severe kidney malfunction, diabetes, coeliac disease, allergies/sensitivity (asthma, allergic to BA, eczema, allergic rhinoconjunctivitis), coagulation disorders and first degree relatives with Crohn's disease or type I diabetes mellitus.[2] Pregnant or breastfeeding women, patients with a dysfunctional liver and gallbladder, an active peptic ulcer, biliary colic, gallstone disease, an acute infection of gallbladder or bile ducts or a bile duct obstruction were also excluded. None of the participants were allowed to take oral anticonception, barbiturates, antacid, cholestyramine or colestipol, nitrendipine, cyclosporine, ciprofloxacine, rosuvastatine, non-steroidal anti-inflammatory drugs, proton pump inhibitors and drugs effecting gastric motility for at least 2 weeks prior to the study and during the study. The VSI questionnaire was used to evaluate GI-specific anxiety.[7] The DSS was used to evaluate the dyspeptic symptom intensity over the preceding 3 months in patients with dyspepsia.[8] The patients filled out daily the Leuven Postprandial Distress Scale diary (LPDS) for 14 weeks.[9] This protocol conformed to the 1975 Declaration of Helsinki and was approved by the Leuven University Hospitals Ethics Committee (s59738; 12/07/2017). All participants approved and signed the informed consent before inclusion in this study with ClinicalTrials.gov number NCT03004118 and EudraCT number 2016-003631-37.

5.2.2.2 Double blinded, placebo controlled, randomized trial

The trial started with a two-week drug discontinuation (drugs interfering with UDCA and drugs effecting GI motility), followed by a four week intake of placebo (Laboratoria Wolfs, Zwijndrecht, Belgium) or UDCA (8-10mg/kg body weight/day; ursochol, pharmacy of UZ Leuven, Leuven, Belgium), a washout period of four weeks, and again a four week intake of UDCA or placebo. The participants took 2 or 3 pills a day, depending on their body weight. After each medication period, the participant was asked to come to the hospital for a gastroduodenoscopy, duodenal fluid aspiration and IGP measurement. On this day, no medication was taken. The participants as well as the investigator were blinded for the allocate treatment. The order of medication intake was randomized by an uninvolved third party and unblinded only after all clinical studies were performed.

5.2.2.3 Duodenal mucosal integrity

Gastroduodenoscopy was performed by an experienced endoscopist to obtain ten biopsy samples from the second part of the duodenum using standard biopsy forceps (Radial Jaw3, outside diameter

2.2 mm; Boston Scientific, Natick, Massachusetts, USA).[2] Three biopsy specimens were kept in ice-cold HBSS buffer for Ussing chamber experiments.[10] Biopsies were mounted in modified 3 ml Ussing chambers (Mussler Scientific Instruments, Aachen, Germany).[2] This technique permitted us to measure mucosal integrity. TEER was recorded every 30 min over 2 h.[2] For the results, the average was calculated and presented as $\Omega.cm^2$. The paracellular probe, FITC-dx4 (molecular mass=4000 Da, 1 mg/ml; Sigma-Aldrich, St Louis, Missouri, USA), was added to the mucosal compartment and serosal samples were collected every 30 min over 2 hours.[2] Afterwards, the fluorescence level was measured using a fluorescence reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany) and the passage of FITC-dx4 was presented as pmol.[2]

5.2.2.4 Western blot

Two biopsy samples were snap-frozen in liquid nitrogen. Protein extracts (15µg) were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane.[2] Membranes were incubated overnight with primary antibodies: rabbit anti-TGR5 (1:2000; 72608 Abcam), mouse anti-VDR (1:1000; 89626 Abcam) and rabbit anti-PXR (1:1000; 85451 Abcam). Secondary antibodies used were peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG (both 1:5000; 31460 and 31430, respectively; Thermo Scientific, Leuven, Belgium).[2] As a protein-loading control, all blots were stained with mouse anti-vinculin (1:5000; V9131 Sigma-Aldrich, Overijse, Belgium).[2] Bands were quantified by densitometry using ImageQuant LAS 4000 mini and ImageQuant TL 1D gel analysis software V8.1. Fold change was determined relative to the average of the healthy group.[2]

5.2.2.5 Microbiome analysis

Fecal samples during the placebo, UDCA and washout period were collected by the patients. Four duodenal biopsies per study visit per patient were snapfrozen in liquid nitrogen. Duodenal aspirates during fasting and fed state were collected during the study visits. All samples were stored at -80°C until further processing. Microbial DNA/RNA extraction from biopsies was performed by using the MagAttractPowerMicrobiome DNA/RNA KF Kit (27600-4-KF, Qiagen). DNA extraction from feces and duodenal fluids started with chemical (lysis buffer) and mechanical (cell disruption with glass beads and heatschock) lysis. This was followed by a robotized procedure to isolate DNA from the supernatans. Next, 184 DNA samples (±10ng/µl) were amplified with V4 illumina Miseq DUAL-INDEX-primers. Two plates of 96-dual-index-primer-pairs were used with each primer having a different barcode resulting in 192 different combinations of 2 barcodes. Two positive and two negative controls were present as internal control on each plate. High-throughput-gel-electrophoresis was performed to visualize the PCR amplicons. After evaluation of the PCR amplicons, the PCR was

performed 2 times more to have enough volume and to reduce mismatching. The PCR products were pooled per sample and the concentration was measured by the fragment analyzer (Advanced Analytical Technologies, Germany). Next, 100ng per sample was pooled in a library. This library-pool was cleaned with QIAquick PCR Purification Kit (28106, Qiagen) and the concentration was measured with the Qubit 3 fluorometer (Invitrogen Thermo Scientific). The library was sent to the Nucleomics core for sequencing. Finally, questionnaires about health, mental and physical wellbeing, eating behaviour, medical information, work/studies and lifestyle and blood samples were collected to investigate the intestinal microbiome and evaluate microbial covariants. This technique was performed in collaboration with the Bioinformatics and (Eco)-Systems Biology lab of Prof J. Raes at VIB-KU Leuven (Leuven, Belgium).

5.2.2.6 Eosinophil and mast cell count

One duodenal biopsy was used for eosinophil and mast cell counting. Deparaffinisation and blocking with REAL Peroxidase Blocking (Dako) and Protein Blocking Solution (Dako) was performed on 5 µm sections.[2] Eosinophils were stained by incubation for two hours in mouse anti-eosinophilic major basic protein (MBP) (1:20; AbD Serotec, Kidlington, UK).[2] Mast cells were stained by incubation for 30 min in mouse anti-mast cell tryptase (1:200; Dako).[2] Next, sections were incubated for 30 minutes with secondary horse anti-mouse biotinylated antibody (1:200; Vector Laboratories, Burlingame, California, USA).[2] Diaminobenzidine and Harris's haematoxylin counterstain was used as the chromogen and for visualization of nuclei, respectively. This technique was performed by the Translational Mucosal Immunology lab of Prof M. Vicario at the institut de Recerca Vall d'Hebron (Barcelona, Spain). Eosinophil and mast cells were counted in whole biopsy sections at ×20 magnification in a blinded manner.

5.2.2.7 Duodenal fluid aspirations

After the gastroduodenoscopy, an aspiration catheter was placed in the second part of the duodenum under fluoroscopic control. Duodenal fluids were aspirated and collected every 15 minutes during 1 hour in the fasted state and during 1.5 hours after an orally ingested mixed liquid meal (Fortimel Energy®; Nutricia, Zoetendaal, The Netherlands, 150 kcal per 100 mL with 5.9 g proteins, 18.4 g carbohydrates and 5.8 g lipids, pH 6.7). Concentration of TCA, GCA, TDCA, GDCA, TUDCA, GUDCA, TCDCA, GCDCA, CA, DCA, CDCA and LCA for each participant and time point were measured with LC-MS/MS.[11] The fasting and fed BA concentrations were calculated as the average of all time points in fasted and fed state, respectively. BA were expressed as absolute concentrations (mM). Total BA was the molar sum of all measured BA.

5.2.2.8 Intragastric pressure measurement

A high-resolution manometry catheter (36-channel HRM) was placed in the gastric fundus to measure IGP.[12] A perfusion tube (OD 2mm) was introduced through the nose and positioned in the stomach. After a stabilization period of 15 minutes, intragastric infusion of the liquid meal started at a constant speed of 60 mL per minute.[11] At 1-minute intervals, the subjects were asked to score their satiation on a graphic rating scale that combines verbal descriptors on a scale graded 0-5.[11] At 5-minute intervals the volunteers were asked to fill out visual analogue scales for 11 dyspeptic symptoms.[11] The intragastric infusion was stopped as soon as the volunteers scored maximally on one of the epigastric symptoms or when a score of 5 was reached on the satiety scores.[11]

5.2.3 Statistical Analysis

We used GraphPad Prism 7.04 to analyze the effect of UDCA on multiple parameters in this *in vitro* and *in vivo* experiment. Values were considered significantly different when $p<0.05$. As we have two different categorical independent variables (condition & time) influencing one continuous variable (TEER, IGP, dyspeptic symptom scores …), we used the repeated measures Two-Way-ANOVA with Bonferroni-correction for multiple testing.[5] Also, two-tailed unpaired t-tests or Mann–Whitney U tests, depending on data distribution, were performed to analyze between-group differences. Pearson or spearman correlation coefficient was used, where appropriate, for correlation analysis. Data were presented as mean±SEM or as median (IQR). Regarding the microbiota data, we used Rstudio and the phyloseq library to evaluate the results.

5.3 Results

5.3.1 Effect of ursodeoxycholic acid on mucosal integrity *in vitro*

5.3.1.1 High concentrations of ursodeoxycholic acid increased cell viability

We used cell Proliferation Reagent WST-1 to quantify cell proliferation, growth, viability and chemosensitvity in cell populations.[5, 13] We observed that exposure to 10mM UDCA increased absorbance of the caco-2 cells at the three different wavelengths ($p<0.0001$ at 440nm, 470nm and 600nm). At wavelength 440 and 600, exposure to FaHIFpool HV + 5mM UDCA, FaHIFpool HV + 10mM UDCA and FaHIFpool FD + 10mM UDCA also increased the absorbance of the caco-2 cells ($p<0.05$).

5.3.1.2 Duodenal aspirates affect transepithelial electrical resistance in vitro

We calculated the percentage change of TEER per condition over time, setting the baseline value as 100% (Figure 5.1).[5] To determine how the TEER values were affected by condition, we compared all conditions to the control ($HBSS^+$) and with each other.[5] Our results showed that TEER of caco-2 cells significantly change when exposed to FaHIFpool FD+UDCA and FaHIFpool HV±UDCA compared to the control condition ($p<0.01$), while TEER of caco-2 cells exposed to UDCA was similar to the TEER of the control condition ($p>0.05$). Time and interaction also explained partially the variability of TEER between the conditions. Furthermore, addition of UDCA to FaHIFpool FD and HV had a significantly different effect on TEER compared to the respective pool without UDCA ($p<0.05$). When we only compared TEER values between control condition and FaHIFpool FD, no differences were found ($p>0.1$). However, TEER values differed significantly between control condition and FaHIFpool HV (<0.01).

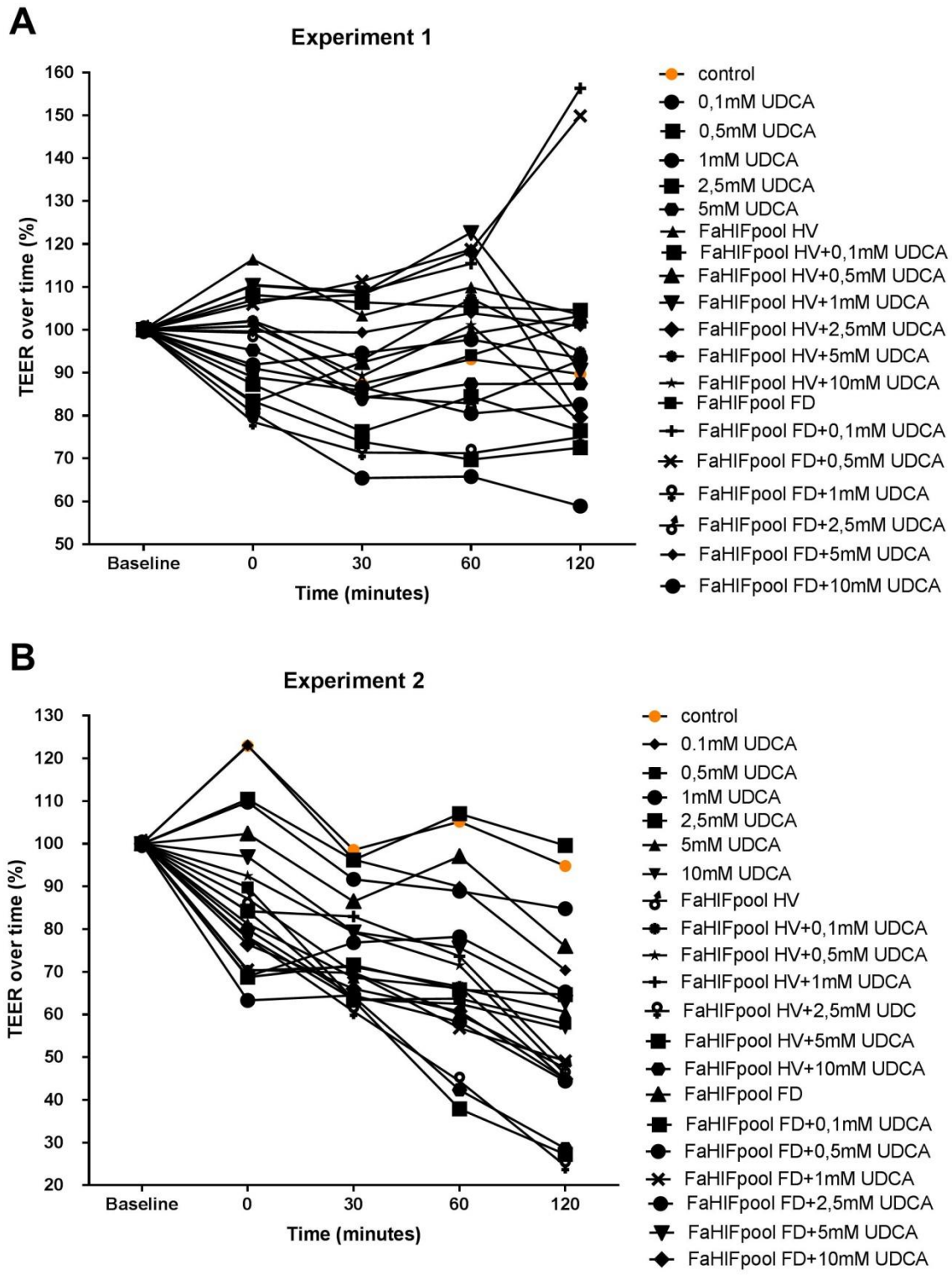


Figure 5.1 The percentage change of the transepithelial electrical resistance of the caco-2 cell monolayers per condition over time with the baseline value as 100%. (A) Experiment one. (B) Experiment two. TEER, transepithelial electrical resistance; UDCA, ursodeoxycholic acid; FaHIFpool HV, pool of fasted human intestinal fluid from healthy volunteers; FaHIFpool FD, pool of fasted human intestinal fluid from patients with functional dyspepsia.

5.3.1.3 FaHIFpool HV and FD differed in individual bile acid concentrations

For each experiment, we pooled duodenal aspirates from patients or HV, resulting in a different FaHIFpool FD and FaHIFpool HV per experiment. In experiment one, FaHIFpool FD showed higher concentrations of GUDCA, TDCA and GDCA compared to FaHIFpool HV ($p=0.02$, $p=0.03$ and $p=0.047$). In experiment two, FaHIFpool FD showed higher concentrations of GUDCA, UDCA, TDCA and GDCA compared to FaHIFpool HV ($p=0.02$, $p=0.04$, $p=0.02$ and $p<0.0001$), while FaHIFpool HV showed higher concentrations of TUDCA, TCA, GCA and TCDCA compared to FaHIFpool FD ($p=0.0043$, $p=0.0006$, $p=0.0006$ and $p=0.0006$).

5.3.2 Effect of ursodeoxycholic acid on duodenal permeability, low-grade inflammation, gut microbiome and meal related sensory motor function in patients with functional dyspepsia

5.3.2.1 Study population

A total of eight patients with FD were recruited (Table 5.1). One patient was classified as EPS, four patients as PDS and three patients belonged to the EPS-PDS overlap group. Five patients fulfilled criteria for co-morbid IBS. Table 5.2 summarizes the grading of dyspeptic symptoms in the patient group during the placebo and UDCA period.

Table 5.1 Demographics and clinical characteristics of the patients with functional dyspepsia

Characteristic	Placebo (N=8)	UDCA (N=8)	P-value
Gender, Female:Male	6:2 (75% female)		-
Age, years (range)	42±5.7 (19-60)		-
BMI, kg m^{-2}	22.8±1.9		-
Dyspeptic symptoms, DSS	9.4±1.3	9.9±1.7	0.47
Gastrointestinal-specific anxiety, VSI	57.3±5.5	56.9±6.2	0.87

Data are mean±SEM; N=8 patients. UDCA, ursodeoxycholic acid; BMI, body mass index; DSS, dyspepsia symptom score; VSI, visceral sensitivity index.

Table 5.2 Frequency of severity grading by DSS for each of the nine dyspeptic symptoms in patients with FD

Symptom	Placebo (N=8)				UDCA (N=8)			
	absent	mild	moderate	severe	absent	mild	moderate	severe
Discomfort	1	4	3	0	1	4	2	1
Postprandial fullness	0	5	2	1	0	4	3	1
Bloating	0	4	4	0	0	5	1	2
Epigastric pain	3	3	2	0	4	1	3	0
Early satiety	1	3	4	0	0	4	4	0
Nausea	1	5	2	0	2	2	3	1
Vomiting	8	0	0	0	8	0	0	0
Belching	1	7	0	0	3	3	2	0
Epigastric burning	4	1	3	0	4	3	1	0

N=8 patients. DSS, dyspepsia symptom score; UDCA, ursodeoxycholic acid; FD, functional dyspepsia.

5.3.2.2 No effect of ursodeoxycholic acid on duodenal permeability in patients with functional dyspepsia

Patients with FD displayed no significant change in TEER and paracellular passage after four weeks of placebo and UDCA (26.1±2.5 $\Omega.cm^2$ vs. 21.4±1.9 $\Omega.cm^2$, p=0.18 and 20.5±2.8 pmol vs. 31.5±4.8 pmol, p=0.09, respectively; Figure 5.2).

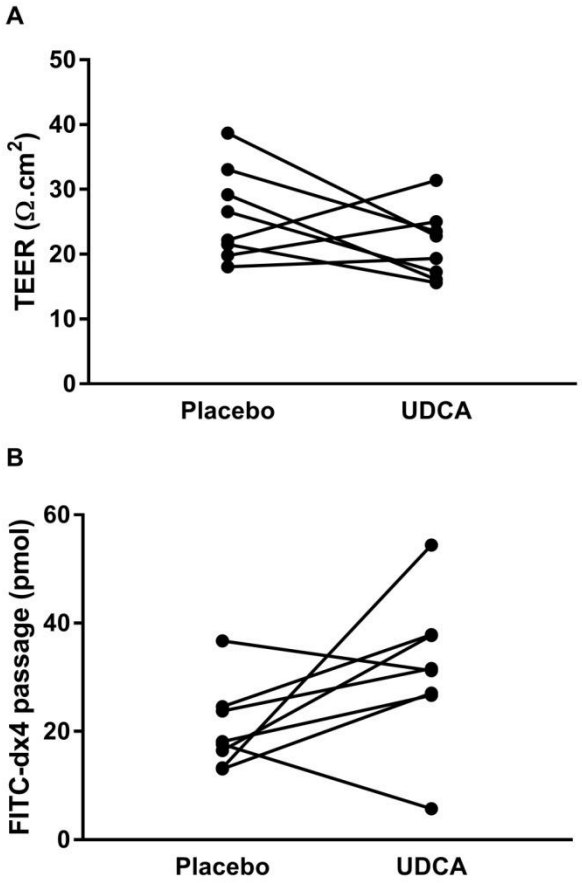


Figure 5.2 ursodeoxycholic acid had no influence on duodenal permeability in patients with functional dyspepsia. Duodenal integrity of patients with FD during placebo and UDCA intake was evaluated in Ussing chambers by measuring TEER (A) and passage of FITC-dx4 (B) of three duodenal biopsy specimens per subject per study visit; N=8. Data are mean. FITC-dx4, fluorescently labelled dextran; TEER, transepithelial electrical resistance; UDCA, ursodeoxycholic acid.

5.3.2.3 Intake of ursodeoxycholic acid influenced the bile acid pool

Intake of UDCA altered the BA pool at the individual BA level compared to placebo. GUDCA and TUDCA luminal BA concentrations increased (2.68±0.61mM vs. 0.18±0.03mM, p<0.0001 and 0.29±0.06mM vs. 0.04±0.005mM, p=0.0009, respectively) after four weeks of UDCA intake compared to placebo (Figure 5.3). The total BA concentration was not altered by UDCA intake compared to placebo (8.67±1.62mM vs. 9.79±1.7mM, p=0.53).

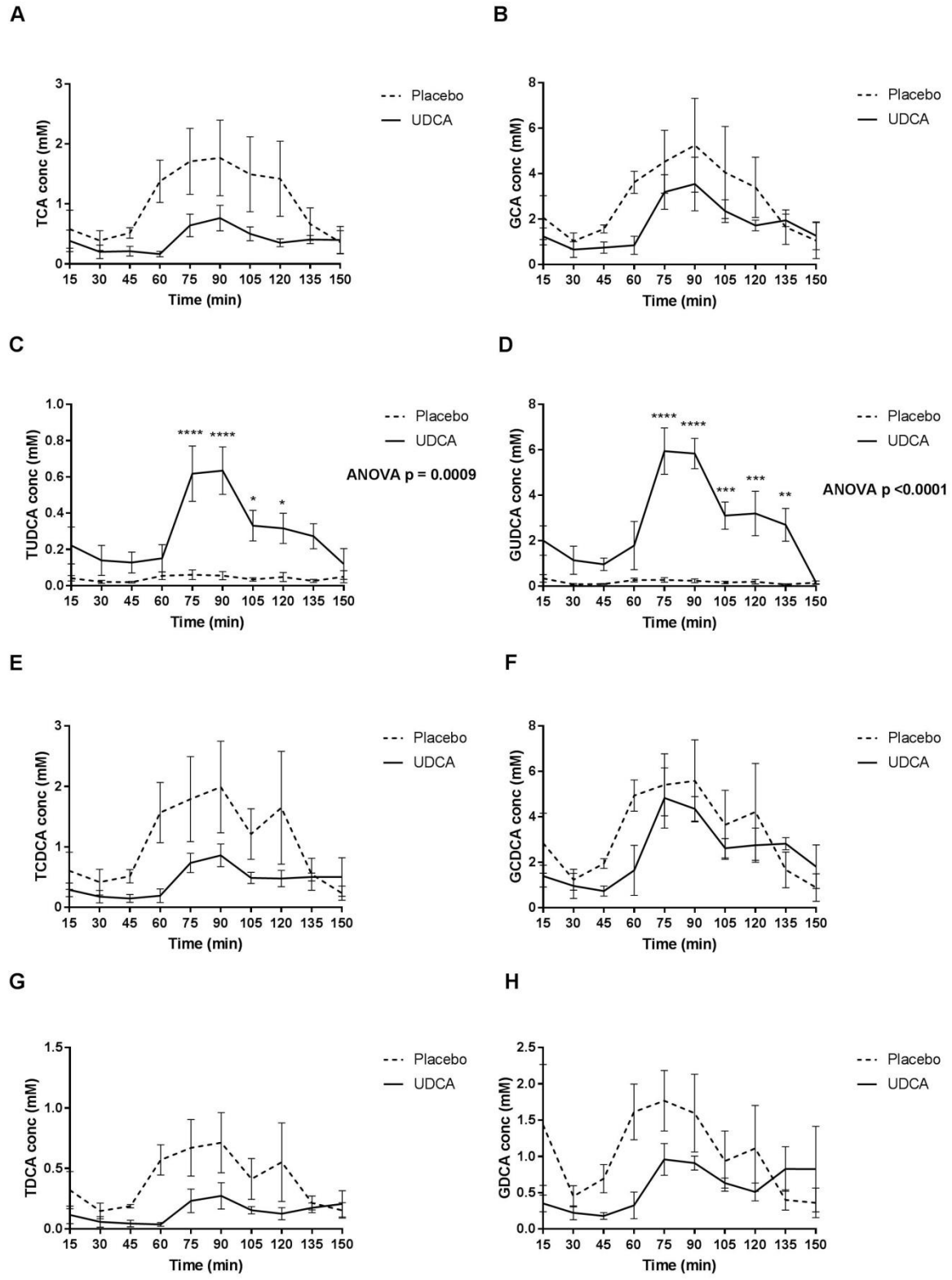
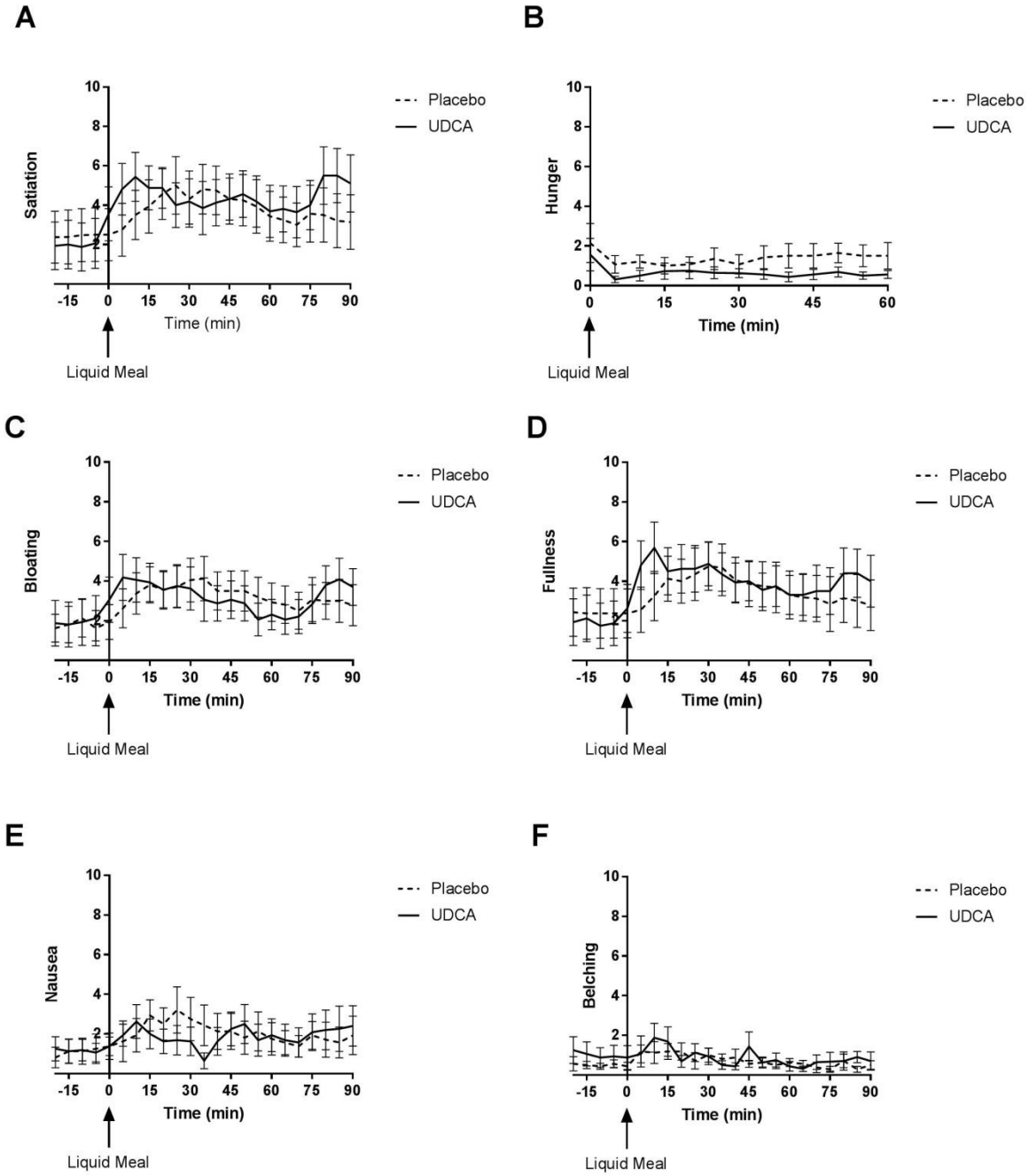


Figure 5.3 Ursodeoxycholic acid intake increased glycoursodeoxycholic acid and tauroursodeoxycholic acid luminal bile acid concentrations. (A-B, E-H) TCA, GCA, TCDCA, GCDCA, TDCA and GDCA concentrations were not statistically different between UDCA and placebo intake. (C&D) GUDCA and TUDCA luminal BA concentrations were increased after four weeks of UDCA intake compared to placebo. N=5. Data are mean±SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. TCA, taurochenodeoxycholic acid; GCA, glycochenodeoxycholic acid;

TUDCA, tauroursodeoxycholic acid; GUDCA, glycoursodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; TDCA, taurodeoxycholic acid; GDCA, glycodeoxycholic acid; UDCA, ursodeoxycholic acid.

5.3.2.4 No effect of ursodeoxycholic acid on dyspeptic symptom occurrence and severity in patients with functional dyspepsia

There was no difference in dyspeptic symptom occurrence and gastro-intestinal specific anxiety between placebo and UDCA during a period of four weeks. There was also no difference in dyspeptic symptom occurrence and severity during an acute experiment with liquid meal intake (Figure 5.4).



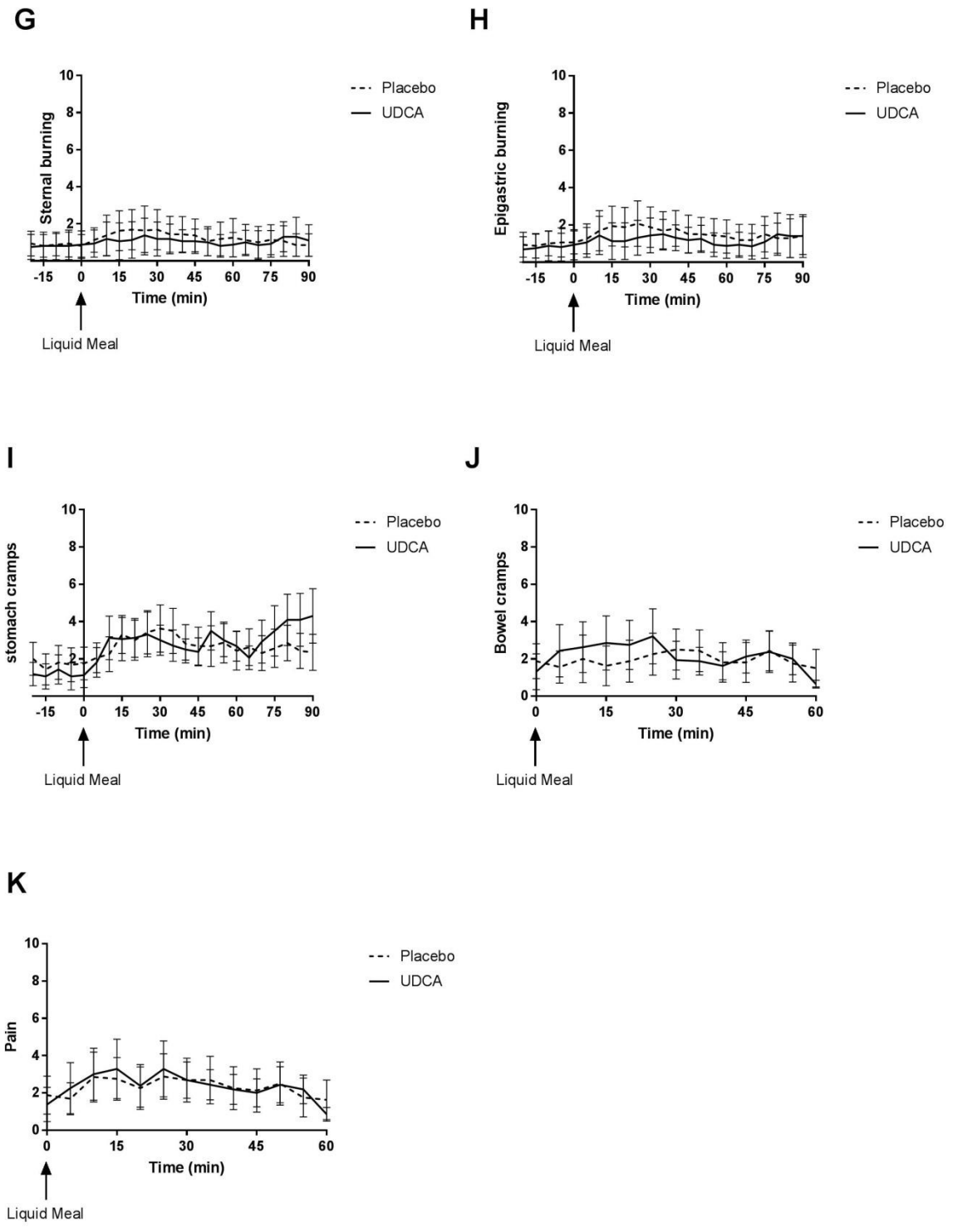


Figure 5.4 Ursodeoxycholic acid had no influence on dyspeptic symptom occurrence during and after liquid meal intake in patients with functional dyspepsia. (A) Satiation, (C) Bloating, (D) Fullness, (E) Nausea, (F) Belching, (G) Sternal burning, (H) Epigastric burning and (I) Stomach cramp scores before and after liquid meal intake after four weeks of placebo and UDCA intake. (B) Hunger, (J) Bowel cramps and (K) Epigastric pain scores after liquid meal intake after four weeks of placebo and UDCA intake. No differences were found in dyspeptic symptom occurrence between placebo and UDCA intake; N=8. UDCA, ursodeoxycholic acid.

5.3.2.5 Ursodeoxycholic acid influenced the correlation between bile acid and duodenal permeability and symptom occurence

After four weeks of placebo intake, no correlations between BA and duodenal permeability remained significant after correction for multiple testing. However, total BA concentrations during fed state correlated negatively with paracellular passage (p=0.003) after four weeks of UDCA intake (Table 5.3). Furthermore, fed GCA concentrations correlated with discomfort (p≤0.005) after four weeks of placebo and UDCA intake, while fed GDCA concentrations correlated with epigastric pain and epigastric burning after four weeks of placebo intake (both p=0.005; Table 5.5).

Table 5.3 Correlation between clinical factors and total bile acids in patients with functional dyspepsia

Clinical factor	Placebo				UDCA			
	Fasted state		Fed state		Fasted state		Fed state	
	P-value	Correlation coefficient	P-value	Correlation coefficient	P-value	Correlation coefficient	P-value	Correlation coefficient
TEER	0.39	-0.50	0.33	0.55	0.46	0.43	0.81	0.15
Paracell. passage	0.40	-0.49	0.56	0.35	0.65	-0.28	**0.003**	**-0.98**
Discomfort	0.08	-0.83	**0.03**	**0.92**	0.89	0.09	0.07	0.85
Postprandial fullness	0.38	-0.51	0.40	0.49	0.26	-0.63	0.95	0.04
bloating	0.67	-0.27	0.73	0.21	0.22	-0.67	0.65	0.28
Epigastric pain	0.09	-0.82	0.06	0.86	0.60	-0.32	0.35	0.54
Early satiation	0.89	-0.08	0.72	0.22	0.41	-0.49	0.40	-0.49
Nausea	0.41	0.49	0.89	-0.09	0.15	-0.74	0.53	0.38
Belching	0.80	0.16	0.78	0.18	0.95	-0.04	**0.03**	**0.91**
Epigastric burning	0.09	-0.82	0.06	0.86	0.81	-0.15	0.50	0.41

Only the correlation between paracellular passage and total BA during fed state after UDCA intake remained significant after correction for multiple testing. N=5 patients. UDCA, ursodeoxycholic acid; TEER, transepithelial electrical resistance.

Table 5.4 Correlation between individual bile acids and clinical symptoms in patients with FD during fasting				
	Placebo		UDCA	
Correlation	**P-value**	**correlation coefficient**	**P-value**	**correlation coefficient**
GUDCA vs TEER	**0.008**	**-0.96**	0.37	0.52
GDCA vs Paracellular passage	0.95	-0.1	0.05	-0.88
Primary BA vs Paracellular passage	0.46	-0.44	0.57	-0.34
GCA vs Discomfort	0.20	-0.80	0.56	0.35
TCA vs Discomfort	0.6	-0.45	0.77	0.21
Secondary BA vs Discomfort	**0.03**	**-0.91**	0.97	-0.02
Primary BA vs Discomfort	0.18	-0.71	0.79	0.03
GUDCA vs Postprandial fullness	**0.048**	**-0.88**	0.30	-0.58
GCA vs Epigastric pain	**0.05**	**-0.92**	0.96	0.03
GDCA vs Epigastric pain	0.1	-0.89	0.76	0.19
GUDCA vs Early satiation	**0.04**	**-0.89**	0.56	-0.35
TDCA vs Early staiation	0.86	0.11	0.98	-0.01
TCDCA vs Nausea	**0.03**	**0.91**	0.06	-0.86
TUDCA vs Nausea	0.046	0.88	0.05	-0.88
GCA vs Epigastric burning	0.05	-0.92	0.87	0.10
GDCA vs Epigastric burning	0.1	-0.89	0.95	-0.04

Only a part of the correlation data is shown. No correlation remained significant after Bonferroni correction for multiple testing. N=5 patients. FD, functional dyspepsia; UDCA, ursodeoxycholic acid; TEER, transepithelial electrical resistance; BA, bile acid; GUDCA, glycoursodeoxycholic acid; GDCA, glycodeoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; TUDCA, tauroursodeoxycholic acid.

Table 5.5 Correlation between individual bile acids and clinical symptoms in patients with FD during fed state				
	Placebo		UDCA	
Correlation	**P-value**	**correlation coefficient**	**P-value**	**correlation coefficient**
GUDCA Vs TEER	0.77	-0.18	0.86	-0.11
GDCA Vs Paracellular passage	0.87	0.10	**0.03**	**-0.91**
Primary BA vs Paracellular passage	0.55	0.36	**0.0124**	**-0.95**
GCA vs Discomfort	**0.0036**	**0.98**	**0.005**	**0.97**
TCA vs Discomfort	**0.018**	**0.94**	0.57	0.35
Primary BA vs Discomfort	0.16	0.74	0.33	0.56
Secondary BA vs Discomfort	**0.02**	**0.94**	**0.048**	**0.88**
GUDCA vs Postprandial fullness	0.51	-0.39	0.33	0.55
GCA vs Epigastric pain	**0.04**	**0.90**	**0.02**	**0.93**
GDCA Vs Epigastric pain	**0.005**	**0.98**	0.7	-0.30
GUDCA vs Early satiation	0.86	-0.11	0.07	0.85
TDCA vs Early satiation	0.53	0.38	**0.01**	**-0.96**
TCDCA vs Nausea	0.73	0.21	0.38	-0.53
TUDCA vs Nausea	0.47	0.43	0.14	-0.75
GCA vs Epigastric burning	**0.04**	**0.90**	0.13	0.76
GDCA vs Epigastric burning	**0.005**	**0.98**	0.81	0.15

Only a part of the correlation data is shown. However, only the correlations between GCA and discomfort during placebo and UDCA intake, GDCA and epigastric pain and GDCA and epigastric burning during placebo intake remained significant after Bonferroni correction for multiple testing. N=5 patients. FD, functional dyspepsia; UDCA, ursodeoxycholic acid; TEER, transepithelial electrical resistance; BA, bile acid; GUDCA, glycoursodeoxycholic acid; GDCA, glycodeoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; TUDCA, tauroursodeoxycholic acid.

5.3.2.6 Ursodeoxycholic acid had no influence on mast cell and eosinophil numbers in duodenal mucosal biopsies of patients with functional dyspepsia

The influence of UDCA on duodenal low-grade inflammation in patients with FD was evaluated by staining for eosinophils and mast cells. Compared with placebo, patients with FD showed similar eosinophil (60.1±17.0 vs 103.4±54.2 MBP+ cells/mm^2, p=0.84; Figure 5.5A) and mast cell (120.5±37.8 vs 111.7±20.2 tryptase+ cells/mm^2, p=0.81; Figure 5.5B) counts after four weeks of UDCA.

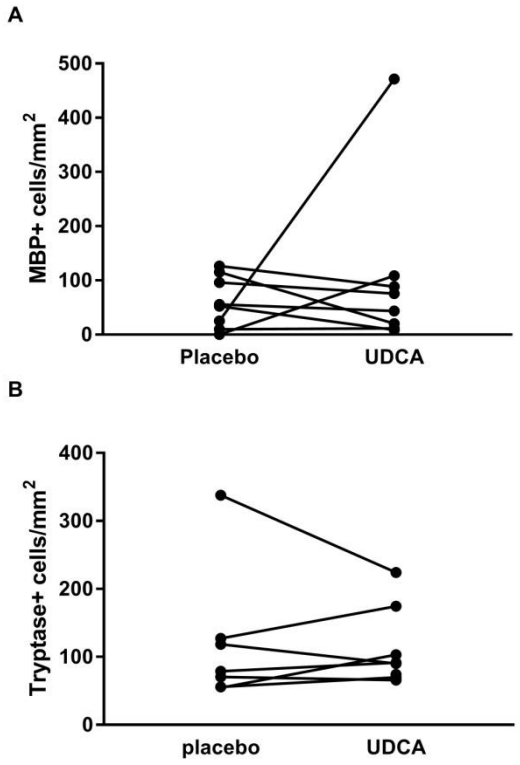


Figure 5.5 Ursodeoxycholic acid had no influence on mast cell and eosinophil numbers in duodenal mucosal biopsies of patients with functional dyspepsia. Duodenal biopsy sections from patients with FD during placebo and UDCA intake were stained for eosinophils using eosinophilic MBP and for mast cells using tryptase.[2] Cells positive for MBP and tryptase were counted in the whole biopsy; N=8. Data are mean. MBP, major basic protein; UDCA, ursodeoxycholic acid.

5.3.2.7 Ursodeoxycholic acid had no influence on duodenal bile acid receptor expression in patients with functional dyspepsia

Duodenal VDR, PXR and TGR5 protein expression did not differ significantly in patients with FD between placebo and UDCA intake (0.7±0.1-fold; p=0.24, 0.7±0.1-fold; p=0.44 and 0.8±0.2-fold; p=0.7, respectively; Figure 5.6).

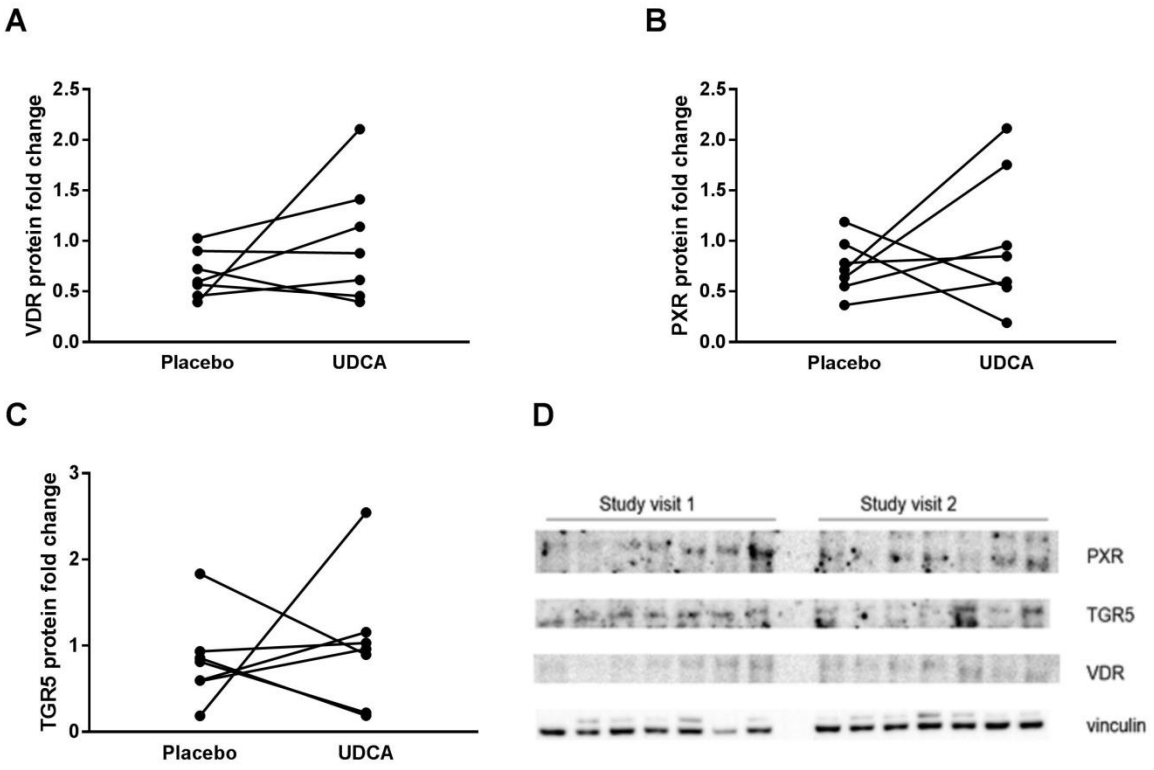


Figure 5.6 Ursodeoxycholic acid had no influence on duodenal bile acid receptor expression in patients with functional dyspepsia. Duodenal VDR, PXR, TGR5 and housekeeping gene vinculin protein expression were evaluated by western blot. Results were normalized to the housekeeping protein, vinculin, and protein fold change was determined relative to the mean value of the control group.[2] (A-C) Duodenal VDR, PXR and TGR5 protein fold change in patients with FD was similar during placebo and UDCA intake; N=7. (D) Representative western blot of seven patients with FD during placebo and during UDCA intake. VDR, vitamin D receptor; PXR, pregnane X receptor; TGR5, transmembrane G-protein-coupled receptor 5; UDCA, ursodeoxycholic acid.

5.3.2.8 No effect of ursodeoxycholic acid on intragastric pressure in patients with functional dyspepsia

IGP measurement was not significantly different in patients with FD between placebo and UDCA intake (p=0.89; Figure 5.7). The volume of liquid meal that was tolerated during the experiment by the patients was also similar after placebo and UDCA intake (137.9±34.8ml vs 164.3±42.24ml; p=0.39).

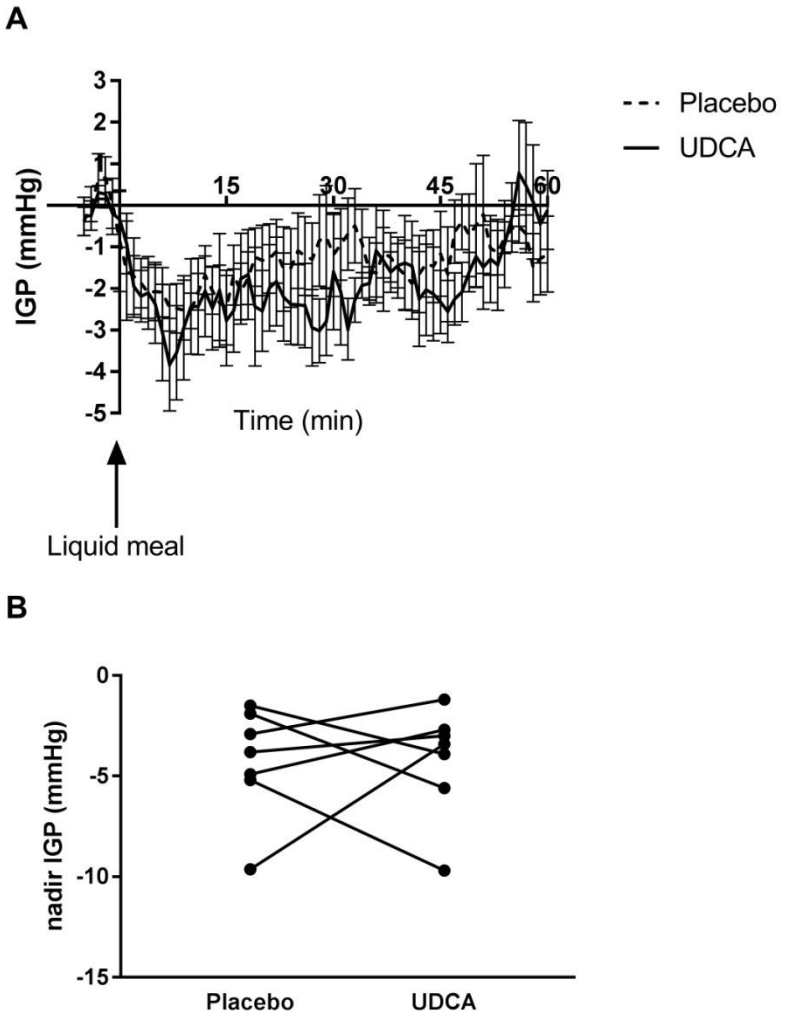


Figure 5.7 Ursodeoxycholic acid had no influence on intragastric pressure in patients with functional dyspepsia. (A) Intragastric pressure measurement curve before and after liquid meal intake after four weeks of placebo and UDCA intake. No differences were found between placebo and UDCA. (B) Nadir IGP or the minimal IGP score during liquid meal intake after four weeks of placebo and UDCA. Again, no differences were found; N=8. UDCA, ursodeoxycholic acid; IGP, intragastric pressure.

5.3.2.9 Intestinal microbiota in patients with functional dyspepsia

These data are incompletely analyzed. As our sample size is too small and the number of multiple testing high, we will only describe our results regarding the gut microbiome in patients with FD with and without UDCA intake, without formal statistical testing (Table 5.6).

Concerning the fecal samples, the microbial interindividual variation explained 57% of the variation ($p<0.001$) between all fecal samples. Abundant genera in the fecal samples were *Bacteriodes*, *Bifidobacterium*, *Blautia*, *Faecalibacterium*, *Roseburia*, *Ruminococcus* and *Prevotella* (Figure 5.8). The phyla present in the fecal samples were *Actinobacteria*, *Bacteriodetes*, *Firmicutes*, *Lentisphaerae*, *Proteobacteria*, *Tenericutes* and *Verrucomicrobia* (Figure 5.9). We noted some individual alterations in genera possibly related to UDCA intake, such as the appearance of *Lactococcus* after four days of UDCA intake in patient IH (Figure 5.10). Patient IF displayed a visually lower abundancy of *Faecalibacterium* and a higher abundancy of *Bifidobacterium* after four days of UDCA intake.

Table 5.6 Number of patients with FD from which molecular data is available

Sample size (N)	Placebo	UDCA
Feces	6	5
Duodenal aspirates	6	6
Duodenal biopsies	2	5

UDCA, ursodeoxycholic acid.

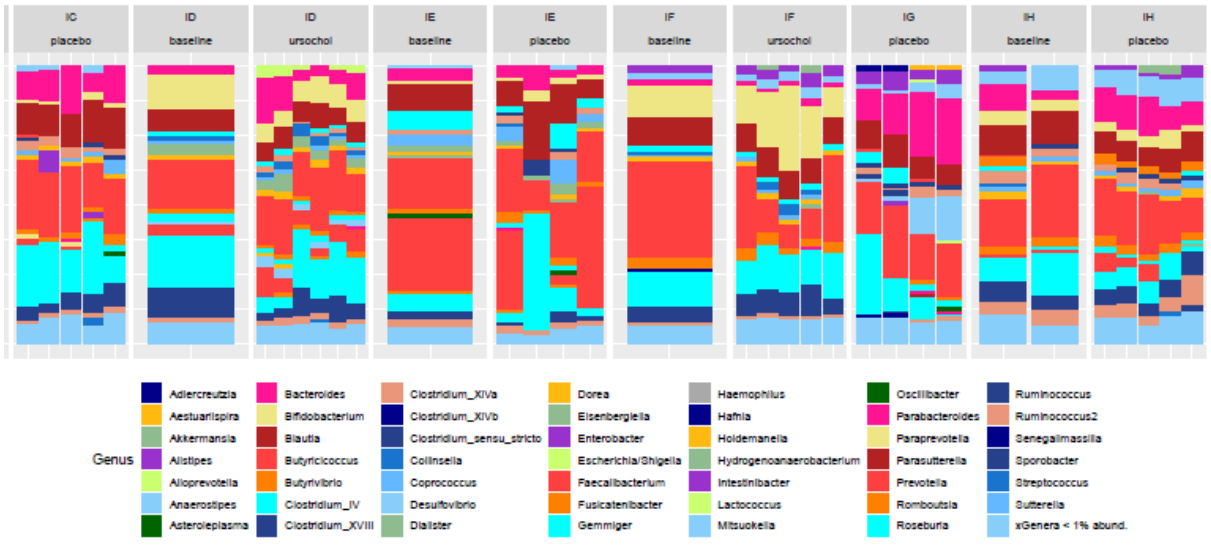


Figure 5.8 Graph displaying the bacteria genera in the fecal samples from several patients (patient IC, ID, IE, IF, IG and IH) during the baseline, placebo and UDCA period (not all data is shown). Most abundant genera were *Bacteriodes, Bifidobacterium, Blautia, Faecalibacterium, Roseburia, Ruminococcus and Prevotella*. The different columns per patient are the different fecal samples taken on different time points during the placebo or UDCA period. Urscohol, UDCA.

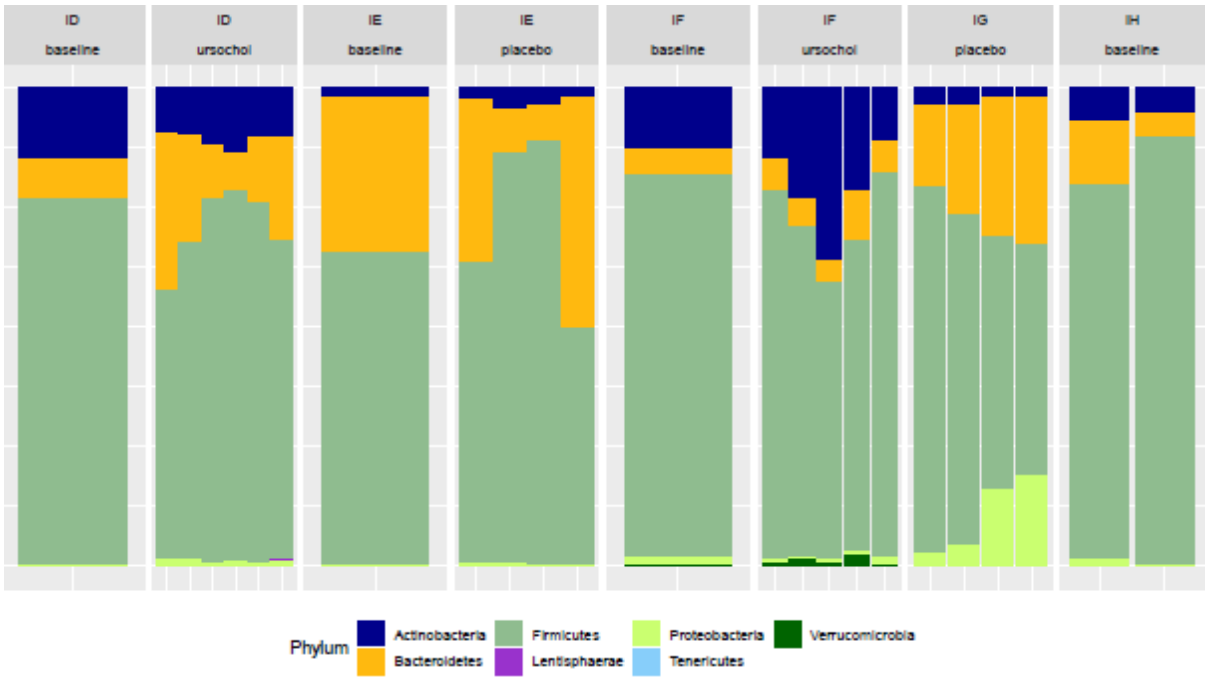


Figure 5.9 Graph displaying the phyla in the fecal samples from several patients (patient ID, IE, IF, IG and IH) during the baseline, placebo and UDCA period (not all data is shown). *Firmicutes* was predominantly present in fecal samples. The other phyla present were *Actinobacteria, Bacteriodetes, Lentisphaerae, Proteobacteria, Tenericutes* and *Verrucomicrobia*. The different columns per patient are the different fecal samples taken on different time points during the placebo or UDCA period. Urscohol, UDCA.

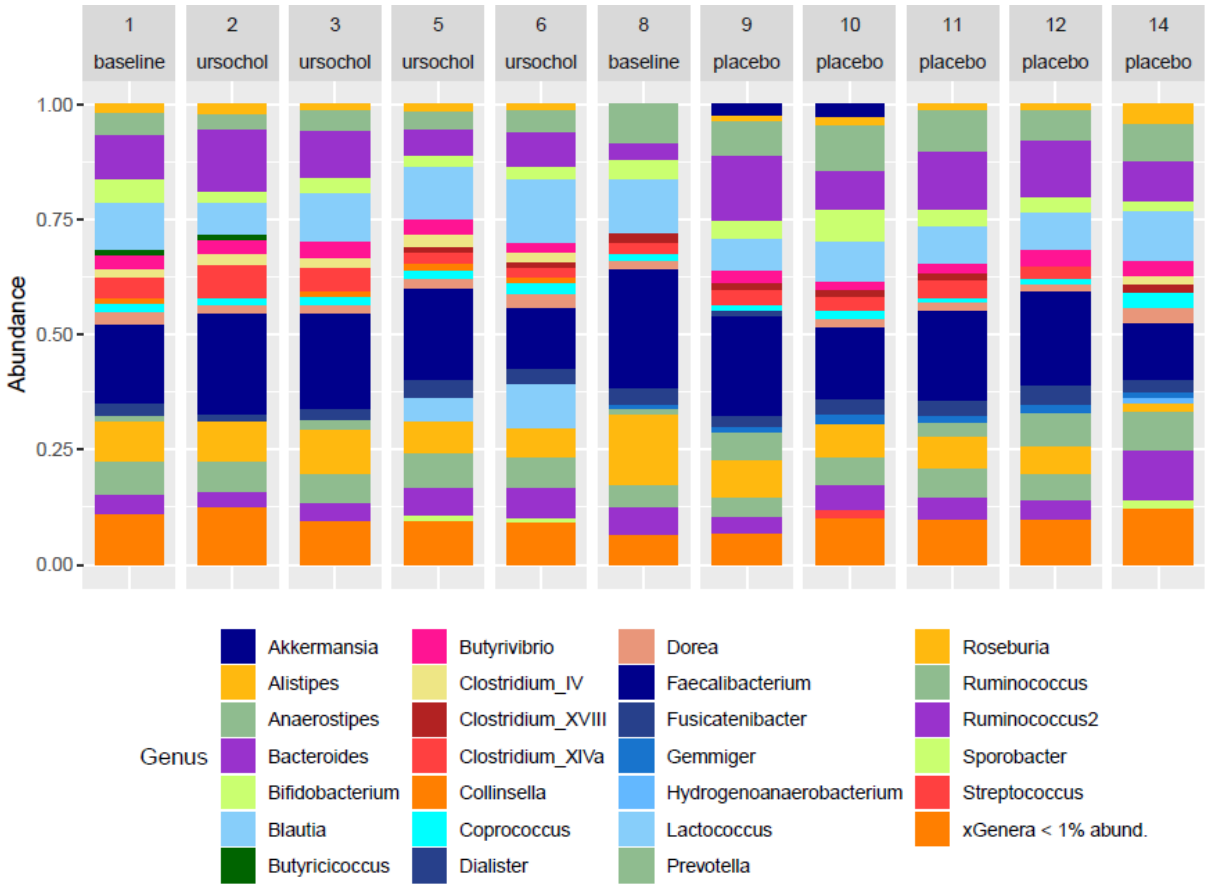


Figure 5.10 Graph displaying the genera in the fecal samples from patient IH during the placebo and UDCA period. An interesting observation was the appearance of *Lactococcus* after four days of UDCA intake in this particular patient. Every column (1-14) is a fecal sample taken on a different time point. Urscohol, UDCA.

Concerning the duodenal aspirates, most abundant genera were *Bacteriodes, Haemophilus, Prevotella, Rothia, Streptococcus* and *Veillonella* (Figure 5.11). The phyla present in the duodenal aspirates were *Actinobacteria, Bacteriodetes, Deinococcus-Thermus, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Synergistetes, Tenericutes* and *Verrucomicrobia* (Figure 5.12). Comparing the different genera present in duodenal aspirates from the same patient during the placebo and UDCA period, some alterations were present. For patient IF, we noted that during the placebo period, *Escherichia/Shigella* was predominantly present in the duodenal fluid. However, during the UDCA period, *Streptococcus* was predominant.

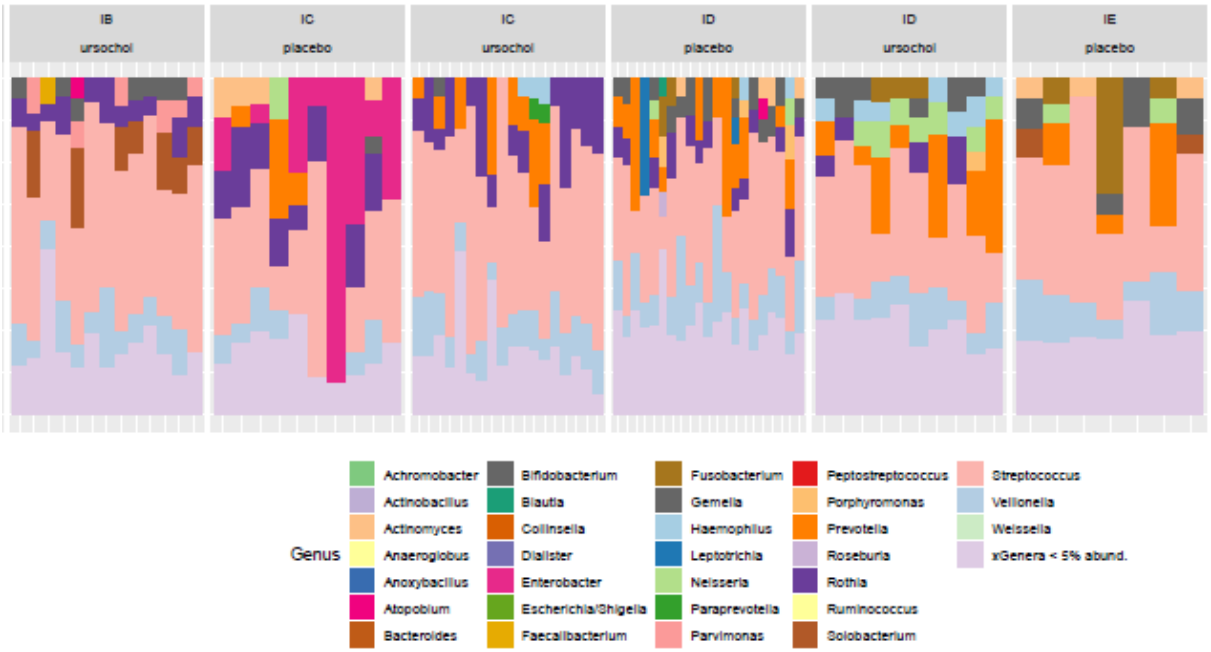


Figure 5.11 Graph displaying the genera in the duodenal aspirates from several patients (patient IB, IC, ID and IE) during the placebo and UDCA period (not all data is shown). Abundant genera *were Bacteriodes, Haemophilus, Prevotella, Rothia, Streptococcus* and *Veillonella*. The different columns per patient are the different duodenal aspirates collected (on fixed time points during fasted and fed state) during the placebo or UDCA period. Urscohol, UDCA.

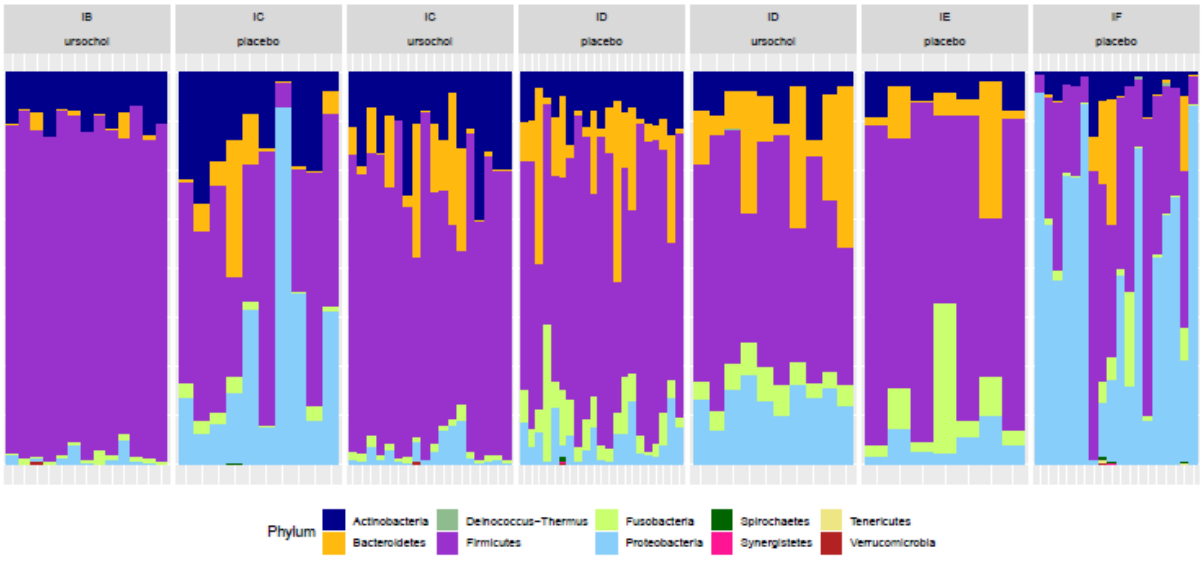


Figure 5.12 Graph displaying the phyla in the duodenal aspirates from several patients (patient IB, IC, ID, IE and IF) during the placebo and UDCA period (not all data is shown). The phyla present in the duodenal aspirates were *Actinobacteria, Bacteriodetes, Deinococcus-Thermus, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Synergistetes, Tenericutes* and *Verrucomicrobia*. The different columns per patient are the different duodenal aspirates collected (on fixed time points during fasted and fed state) during the placebo or UDCA period. Urscohol, UDCA.

Concerning the duodenal biopsies, the genus *Streptococcus* and the phylum *Firmicutes* were predominantly present in the duodenal mucosa. Other abundant genera were *Rothia, Veillonella, Prevotella, Actinomyces, Enterobacter, Neisseria* and *Haemophilus* (Figure 5.13). The other phyla present were *Actinobacteria, Bacteriodetes, Deinococcus-Thermus, Fusobacteria, Proteobacteria, Spirochaetes, Tenericutes* and *Verrucomicrobia* (Figure 5.14).

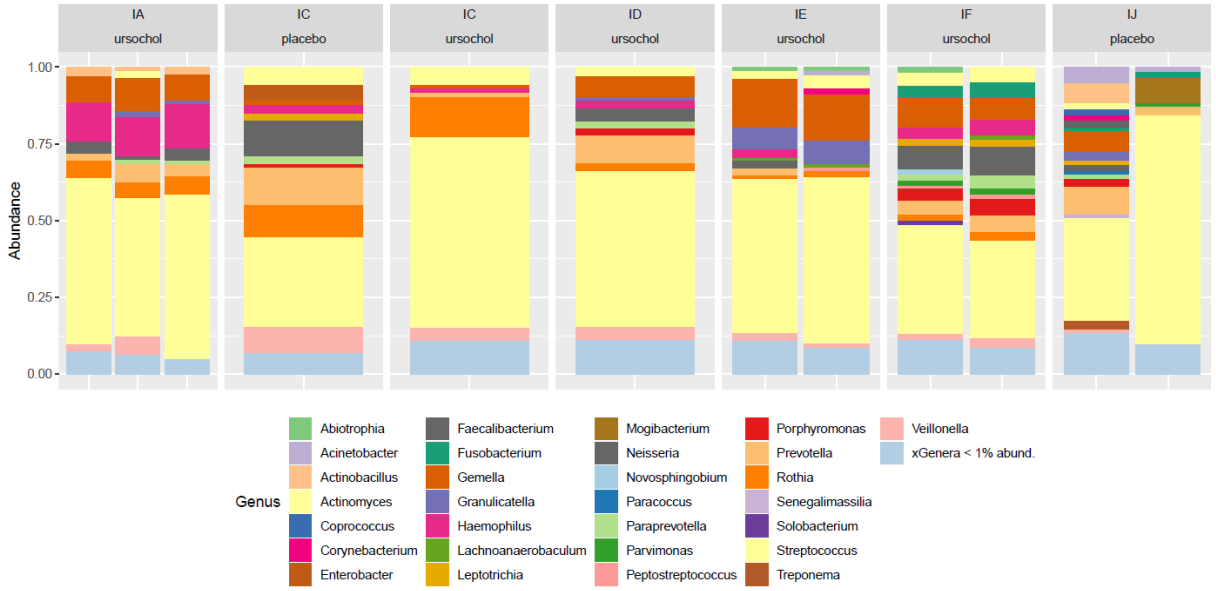


Figure 5.13 Graph displaying the genera in the duodenal biopsies from patients (patient IA, IC, ID, IE, IF and IJ) during the placebo and UDCA period. *Streptococcus* was predominantly present and other abundant genera were *Rothia, Veillonella, Prevotella, Actinomyces, Enterobacter, Neisseria* and *Haemophilus*. The different columns per patient are the different biopsies collected at the same time during the placebo or UDCA period. Urscohol, UDCA.

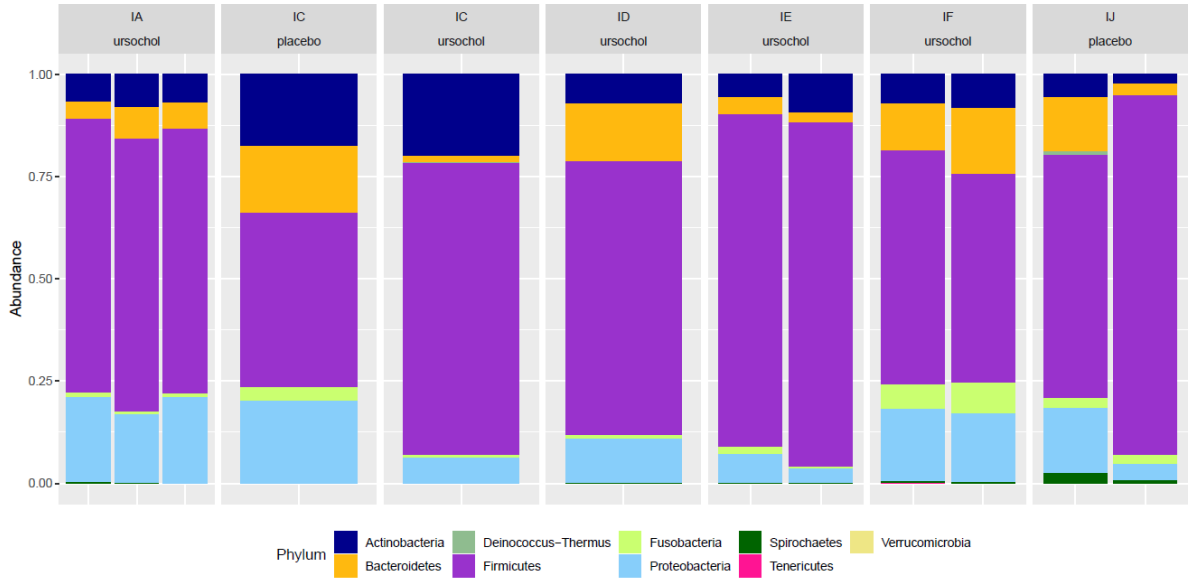


Figure 5.14 Graph displaying the phyla in the duodenal biopsies from patients (patient IA, IC, ID, IE, IF and IJ) during the placebo and UDCA period. *Firmicutes* was predominantly present in the duodenal biopsies. The other phyla present were *Actinobacteria, Bacteriodetes, Deinococcus-Thermus, Fusobacteria, Proteobacteria, Spirochaetes, Tenericutes* and *Verrucomicrobia*. The different columns per patient are the different biopsies collected at the same time during the placebo or UDCA period. Urscohol, UDCA.

The genera and phyla present in duodenal biopsies and aspirates were quite similar, while the microbiota present in the feces were different, consistent with the Principal coordinative analysis (PCoA) on Bray-Curtis dissimilarity distances at genus level (Figure 5.15).

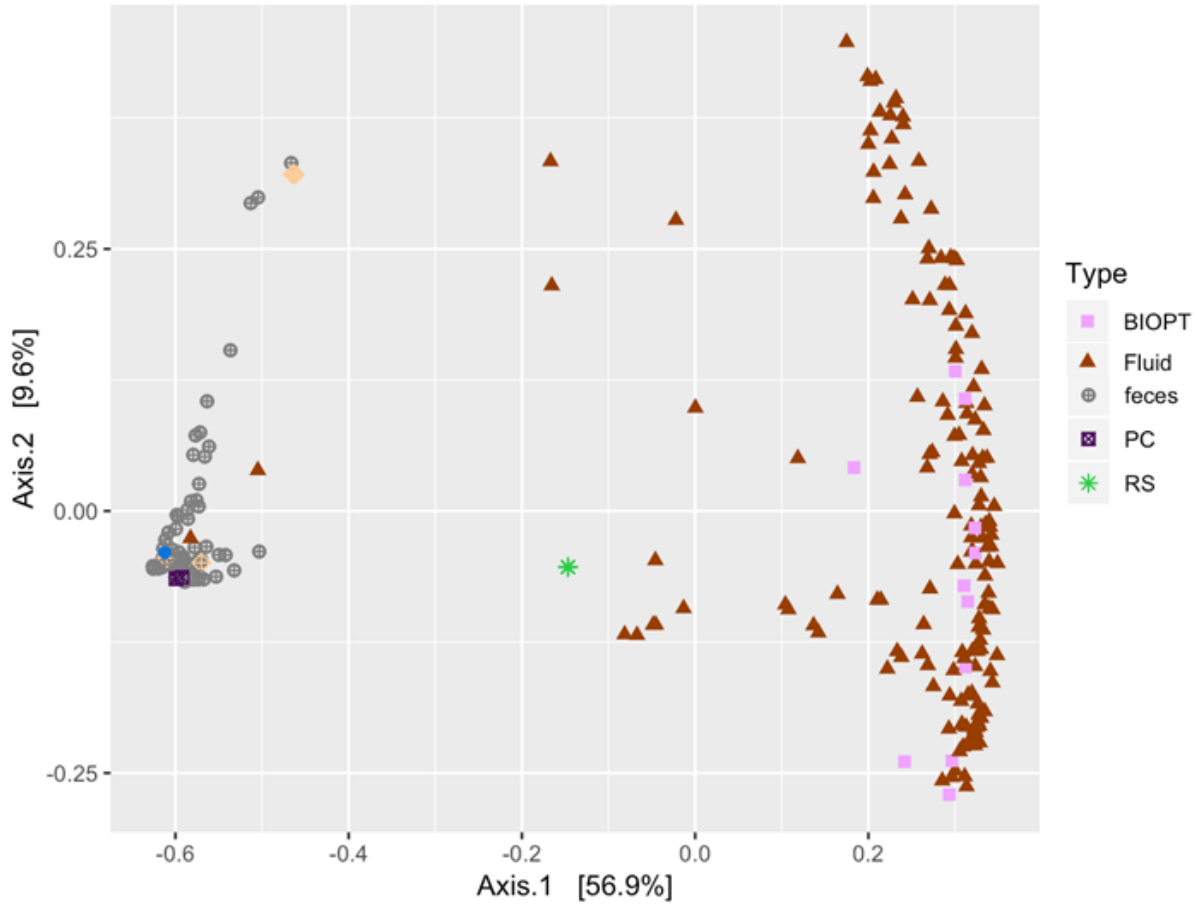


Figure 5.15 Microbial community variation. PCoA of genus-level Bray-Curtis dissimilarity of the bacterial and archael fraction of feces, duodenal biopsies and aspirates. The percentage of variation explained by the two first PCoA dimensions are reported on the axes. BIOPT, biopsies; Fluid, duodenal aspirates; feces, fecal samples; PC, positive control; RS, *Runella* strain.

5.4 Discussion

In the current study, we evaluated UDCA as a potential treatment for patients with FD, based on the potential mucosal protective effects in the duodenum.

We used a cell culture model to study the effect of UDCA on cell viability using WST-1, which allows to visualize cell metabolism activity.[14] We observed that UDCA protects cell viability in a concentration-dependent manner: increasing UDCA concentrations increased cell viability. These results implied that UDCA increases the rate of metabolic activity of the cells, in line with a putative mucosal protective effect and paving the way for our clinical study.

Second, we showed that the aspirated duodenal fluids had a deteriorating influence on mucosal cell integrity. Paradoxically, however, the aspirated duodenal fluids from HV had a more detrimental effect on membrane integrity than aspirated duodenal fluids from patients with FD. In the FaHIFpool in HV, increased concentrations of primary BA are present compared to the FaHIFpool in FD, while the duodenal FaHIFpool in FD showed increased concentrations of secondary BA compared to the FaHIFpool in HV. This possibly reflects the detergent effect of BA on membrane integrity. However, the findings are not in agreement with our initial hypothesis that duodenal fluids of patients with FD would be more deleterious to membrane integrity than duodenal fluids of HV and our assumption that the secondary BA would have more detergent effects on membrane integrity than primary BA as has been suggested in the literature.[15] On the other hand, the FaHIFpool in FD had higher concentrations of UDCA and GUDCA compared to the FaHIFpool in HV, while the FaHIFpool in HV only had higher concentrations of TUDCA. This suggests that the increased presence of UDCA and UDCA conjugates has mild protective effects on membrane integrity *in vitro*. According to the literature, exogenous UDCA has a protective effect through, amongst others, stabilization of the membrane structure and modulation of the intestinal barrier.[16-17]

UDCA is used as a treatment for gallstones and various liver and bile duct inflammatory diseases, and it showed therapeutic potential against diarrhea in mice models.[5, 18] In the current small and probably heterogenous pool of eight patients with FD, four weeks of treatment with UDCA had no significant effect on symptoms, duodenal permeability, immune cell count, BA receptor expression and gastric accommodation measured with IGP. However, these parameters showed strong interindividual variability and, although the sample size is limited, there was no apparent link of these abnormalties with a specific FD subtype (PDS, EPS and PDS/EPS overlap group).

Four weeks of UDCA intake increased the luminal TUDCA and GUDCA concentrations, but failed to alter the total BA concentration. In a previous study by our group with 15 patients with FD, epigastric burning correlated positively with total BA concentrations in patients during fasting and fed state.[19] In the present study, only during the UDCA period, total BA concentrations correlated negatively with paracellular passage; GCA total concentrations correlated positively with gastric discomfort during the UDCA and placebo period; and GDCA concentrations correlated positively with epigastric burning and pain during the placebo period. Nevertheless, UDCA had no significant effect on GCA, GDCA and total BA concentrations and no effect on symptom occurrence/severity compared to the placebo period. Hence, the relevance of these correlations and the mechanism for their apparent emergence is unclear.

Due to the small sample size (eight patients), some missing fecal samples (caused by an irregular defecation), some missing duodenal aspirates (due to the impossibility to place the aspiration catheter in the duodenum of some patients) and due to some missing results, especially from biopsies (due to PCR difficulties), our current data was too limited for statistical analysis of the microbiota data. In view of this limitation, we provided only a broad description regarding the duodenal and fecal microbiome in patients with FD.

The phyla *Firmicutes* (most dominant and diverse phylum), *Bacteroidetes* and *Actinobacteria* predominate the intestinal tract.[20] The intestinal bacterial count ranges between 10^1 -10^3 cfu/mL in the stomach and jejunum, between 10^4 -10^7 cfu/mL in the ileum and between 10^{10}-10^{13} cfu/mL in the colon.[21] Particularly aerobic bacteria are present in the jejunum and anaerobic bacteria in the ileum, implying that the presence of gut microbiota alters depending on its location in the intestinal tract.[20-21] Moreover, rapidly expanding research indicates alterations in the intestinal microbiota of patients with several FGID.[21]

Concerning our fecal samples, most abundant genera were *Bacteriodes, Bifidobacterium, Blautia, Faecalibacterium, Roseburia, Ruminococcus* and *Prevotella*. The phyla present in the fecal samples were *Actinobacteria, Bacteriodetes, Firmicutes, Lentisphaerae, Proteobacteria, Tenericutes* and *Verrucomicrobia*. In one patient, the genus *Lactococcus* appeared after four days of UDCA intake. Another patient displayed a visually lower abundancy of *Faecalibacterium* and a higher abundancy of *Bifidobacterium* after 3 days of UDCA intake. *Bifidobacterium* is one of the genera that are frequently used as probiotic agents for FGID.[22] Probiotic bacteria are implied to be beneficial in human health through, amongst others, intestinal barrier stabilization and immunomodulation.[23] However, the mechanisms underlying these activities stil need to be clarified.[22] *Faecalibacterium prausnitzii* is an

anti-inflammatory butyrate producer with a low prevalence in IBD patients and is suggested to contribute to intestinal homeostasis.[24] Moreover, it was already shown that daily oral administration of UDCA in a colitis mice model enhanced the ratio of *Firmicutes* to *Bacteroidetes* and increased the abundance of *Clostridium cluster XIVa* and *Akkermansia muciniphila*.[25]

Concerning the duodenal aspirates, most abundant genera in our patient group were *Bacteriodes, Haemophilus, Prevotella, Rothia, Streptococcus* and *Veillonella*. The phyla present in the duodenal aspirates were *Actinobacteria, Bacteriodetes, Deinococcus-Thermus, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Synergistetes, Tenericutes* and *Verrucomicrobia*. In one patient, the genus *Escherichia/Shigella* was predominantly present in duodenal fluid during the placebo period and abolished during the UDCA period. In a study by Strati et al, fecal samples from 40 patients with autism and 40 age- and gender-matched HV showed that the commonly believed proinflammatory *Escherichia/Shigella* and *Clostridium cluster XVIII* correlated positively with constipation and were more abundant in the fecal samples of constipated patients than in non-constipated patients with autism.[26]

In a study by Li et al, duodenal and rectal biopsies, feces and duodenal mucus were obtained from nine HV to determine the microbial composition and diversity in the duodenum and the rectum: the phyla *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria*, and *Proteobacteria* were prevalent in the duodenal and rectal biopsies, feces and duodenal mucus; the phyla *Firmicutes* and *Proteobacteria* predominated in duodenal biopsies and mucus, while the phyla *Firmicutes* and *Bacteroidetes* predominated in rectal biopsies and feces; and several rare phyla, including *Spirochaetes* and *Tenericutes* were only detected in duodenal biopsies and mucus.[27] The data of Li et al in HV are in agreement with our data in patients with FD. However, Li et al also published that the most abundant genera in duodenal biopsies were *Acinetobacter, Bacteroides*, and *Prevotella* and in the duodenal mucus were *Prevotella, Stenotrophomonas*, and *Streptococcus*, while anaerobic microbiota, including *Prevotella, Bacteroides* and *Faecalibacterium*, were predominant in rectal biopsies and feces.[27] This is in contrast with our data: the genus *Bacteroides* was not present in the duodenal biopsies, *Acinetobacter* was only seen in one patient with FD and *Streptococcus* was the most abundant genus in the duodenal biopsies; moreover, the genus *Stenotrophomonas* was not present in the duodenal fluid of our patients with FD. Li et al concluded that more genera are present in the duodenal and rectal biopsies than in the feces and duodenal mucus and that duodenal microbial communities are unique and have a higher biological diversity than rectal samples in HV.[27] Differences may be attributable to differences in selected subjects, including health status and nutritional habits.

According to our biopsy data, the genus *Streptococcus* and the phylum *Firmicutes* were predominantly present in the duodenal mucosa. Other abundant genera were *Rothia, Veillonella, Prevotella, Actinomyces, Enterobacter, Neisseria* and *Haemophilus*. The other phyla present were *Firmicutes*, *Actinobacteria, Bacteriodetes, Deinococcus-Thermus, Fusobacteria, Proteobacteria, Spirochaetes, Tenericutes* and *Verrucomicrobia*. In a recently published letter by Zhong et al, genera in the duodenal mucosa of nine patients with FD and nine HV were described: the predominant genus was *Streptococcus,* which is in agreement with our data and the relative abundance of *Streptococcus* correlated negatively with the genera *Prevotella, Veillonella* and *Actinomyces*; the genera *Prevotella, Veillonella* and *Actinomyces* were decreased in patients with FD compared to HV; the microbial community between patients with FD and controls were significantly different.[28] In a study by Rangel et al, feces and colonic biopsies were obtained from patients with IBS and HV.[29] *Bacteroidetes* were predominant in the colonic biopsies, while *Firmicutes, Actinobacteria* and *Proteobacteria* were predominant in the feces.[29] Moreover, microbial abnormalities featuring in IBS are more clearly present in the feces compared to the mucosa.[29] Luminal bacteria are involved in digestion and immunomodulation, while mucosal bacteria are involved in microbe-immune processes.[29] Depending on the location in the GI tract, the functional abilities of bacteria are likely to differ.[30]

Concerning our *in vitro* study, caco-2 cell models are regularly used for *in vitro* representation of the human GI tract, as they provide a reproducible model and spontaneously form a monolayer mimicking the intestinal epithelium.[5, 31] Moreover, studies have shown a high correlation of Caco-2 permeability coefficients with absorption data in humans.[5, 31] However, Caco-2 cells are colonic cancer cells exclusively consisting of enterocytes, resulting in a debatable representation of the in vivo environment of the healthy duodenum.[5] Moreover, Caco-2 cells display tighter apical junctions as compared to the small intestine.[5, 32] The Caco-2 cell model is static and therefore not able to mime the movement of the gut and it does not reflect the bidirectional influence of the BA pool on the gut microbiota.[5, 31] In caco-2 cell layers, parameters such as TEER and proliferation rate have been documented to increase with time and it has been reported that late passage cells may start growing in multilayers, affecting TEER measurements.[5, 31] Therefore, using the Caco-2 cell model is a powerful tool, but *in vitro* to *in vivo* extrapolation has to be done with caution.[5]

Concerning our *in vivo* study, this pilot study is the first UDCA trial performed in patients with FD. However, this trial is limited by a small number of patients, displaying heterogeneity of patients with FD, and a limited four-week treatment duration of UDCA. Based on our pilot data, we suggest a new trial with a larger sample size, a longer treatment duration and a homogenous patient pool characterized by a decreased duodenal permeability since impaired mucosal integrity is most likely not a universal feature in FD. This trial will be necessary to understand the underlying mechanisms of UDCA in the pathophysiology of FD and will decide whether UDCA can be used in the future as a treatment or as part of a treatment for a subgroup of patients with FD.

To summarize, duodenal fluids have a detergent effect on membrane integrity *in vitro*.[5] Increasing concentrations of UDCA trend towards an increase in the metabolic activity of the cells.[5] A protective effect of UDCA in the pathophysiology of FD was not found in this pilot study. However, in the future an adequately powered trial in well characterized patients needs to be performed to fully understand the clinical effect and underlying mechanisms of UDCA.

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Chapter 6

General discussion and future prospects

6 General discussion and future prospects

The Rome III consensus for FGID defined FD as the presence of one or more recurrent or persistent symptoms (postprandial fullness, early satiation, epigastric pain and epigastric burning as cardinal symptoms, but also bloating, belching, vomiting, nausea) in the gastroduodenal region without evidence of structural disease that is likely to explain the symptoms.[1] The prevalence of FD in the general population is as high as 15-20%.[2] FD seems to be a heterogeneous disorder, in which diverse pathophysiological mechanisms are involved.[2] Initially, gastric abnormalities such as impaired gastric accommodation, delayed gastric emptying and gastric hypersensitivity to distension were primarily considered to underlie the generation of dyspeptic symptoms.[2] More recently, it was suggested that duodenal abnormalities such as increased sensitivity to duodenal acid/lipids could also be implicated in the pathophysiology of FD.[2, 3] Early reports confirmed the presence of low-grade inflammatory changes in the duodenal mucosa of FD patients, which has been hypothesized to contribute to symptom generation by inducing altered motor control and visceral hypersensitivity.[2] Moreover, our group recently demonstrated that impaired duodenal barrier function may represent a pathophysiological mechanism in FD, and that this probably relates to low-grade inflammation.[3] To date, the cause of impaired mucosal integrity and low-grade inflammation in FD remains unclear, but likely contributing candidates are genetic factors, stress and luminal factors. The available treatment options for FD are limited in number and effectiveness, which is at least partly explained by the poorly understood pathogenesis.[2] Keeping in mind the high human and economic burden of FD, more effective treatment is badly needed.[2] The aim of this project is to unravel the pathophysiologic role of duodenal luminal factors in FD, more specifically of BA. We hypothesize that increased duodenal BA exposure or a different BA composition, leads to impaired duodenal mucosal integrity in FD, allowing luminal substances to pass through the mucosa and elicit immune responses. This could provoke visceral hypersensitivity and altered gastric motor control, finally resulting in dyspeptic symptom generation. On the other hand, increased duodenal permeability could lead to an increased passage of BA and other luminal factors, resulting in visceral hypersensitivity and/or altered gastric motor control directly or indirectly via low-grade inflammation.

6.1 Do duodenal implications play a key role in the pathophysiology of functional dyspepsia?

In this manuscript, we reported decreased total duodenal BA concentrations during fasting and an increased duodenal VDR expression in FD. we also showed that BA correlate with duodenal permeability and symptom occurrence, and that duodenal BT is less likely to play a role in the pathophysiology of FD.

Despite our efforts, we could not confirm the original hypothesis of an altered BA pool in patients with FD contributing to duodenal changes such as increased permeability and low-grade inflammation, leading to visceral hypersensitivity and an altered gastric motor control, finally resulting in dyspeptic symptoms. This could be due to various factors including the selection of the patient subpopulation with FD, the site of sample collection in the proximal small bowel and the small sample size in all studies, which is a general limitation. It may however also confirm that, although BA levels are different in the fasting state, BA do not play a role in FD.

Furthermore, the duodenal VDR expression is almost 4-fold increased in our patient group. It is conceivable that the upregulation of VDR in FD is part of a mucosal defense against low-grade inflammation. However VDR upregulation may also be due to a vitamin D deficiency present in the patient group, which was not addressed in the present study. In vitro experiments with monolayers, colonic biopsies from patients with ulcerative colitis and colitis mice models demonstrated that vitamin D and VDR play a key role in the mucosal barrier homeostasis by preserving the integrity of junction complexes by abolishing myosin light chain kinase-dependent tight junction dysregulation during colonic inflammation.[4-6] It was also shown by VDR knockout mice, cultured human intestinal epithelial cells and immunohistochemistry that VDR enhances colonic Claudin-2 (a tight junction protein) expression.[6-7] Moreover, serum vitamin D concentration obtained from 230 subjects with ulcerative colitis is inversely correlated with mucosal inflammation, disease activity and proinflammatory cytokines and positively correlated with mucosal VDR expression and epithelial junction proteins.[8] However, in a study by Kühne et al, the tight junction morphology and intestinal permeability for large molecules were not affected in VDR knockout mice compared to wild-type mice.[9]

Considering the state of the art, FD was originally approached as a gastric dysfunction. The cardinal symptoms epigastric pain, epigastric burning, postprandial fullness and early satiation were suggested to relate to delayed gastric emptying or impaired gastric accommodation.[10] However, the lack of consistent findings, correlations between symptoms and physiological abnormalities and efficient therapy caused doubt.[10] A new way of thinking launched the duodenum as the potential source of FD symptoms and became a target of FD research.[10] In view of this, it was shown by Du et al that low-grade inflammation, characterized by local eosinophil and mast cell infiltration, is a pathophysiological mechanism in FD.[11] In addition, ultrastructural alterations in the degranulation state of eosinophils and mast cells were reported.[12] Moreover, Potter et al suggested that PPIs have a beneficial effect in patients with PDS by suppressing duodenal eosinophilia.[13] Low-grade inflammation in patients with FD is associated with dyspeptic symptoms and is believed to contribute to the occurrence of motor-sensory abnormalities and neuroimmuno-dysregulation.[14-16] Furthermore, Vanheel et al, as previously mentioned, reported that patients with FD display an impaired duodenal barrier function characterized by lower TEER, increased paracellular passage and an abnormal expression of cell-to-cell adhesion proteins.[3] Furthermore, they found an association between impaired duodenal barrier function and low-grade inflammation.[3] Ishigami et al confirmed the presence of increased duodenal mucosal permeability in patients with FD.[17] This indicates that FD is not a purely functional disorder as it shows confirmed structural changes in the gastrointestinal tract.[3] On the other hand, Tziatzios et al suggested a causal link between small intestinal bacterial overgrowth and FD.[18] They hypothesized that infectious gastroenteritis may result in an increased proliferation of coliform bacteria, leading to an abnormal fermentation of carbohydrates, resulting in luminal distension, increased intestinal permeability, immune response prolongation and finally dyspeptic symptoms.[18]

Considering our results and the state of the art, we hypothesize further on the potential mechanism of BA in FD. We suggest that, due to the decreased primary to secondary BA ratio, the luminal BA pool has more detergent effects on the mucosal barrier, resulting in impaired duodenal mucosal integrity. An increased mucosal permeability may allow the passage of luminal antigens through the mucosa, evoking immune responses.[3] By nerve-mediated activation by corticotropin-releasing hormone or cholinergic pathways, mucosal mast cells release multiple mediators effecting transcellular and/or paracellular permeability.[19] This may lead to an inappropriate immune activation that in a susceptible host results in mucosal inflammation.[19] During the inflammation, cytokines are released and activate eosinophils.[3] During eosinophil degranulation, amongst others, nerve growth factor and major basic proteins are released and may provoke visceral hypersensitivity and altered gastric motor control, finally resulting in dyspeptic symptom generation.[3, 10]

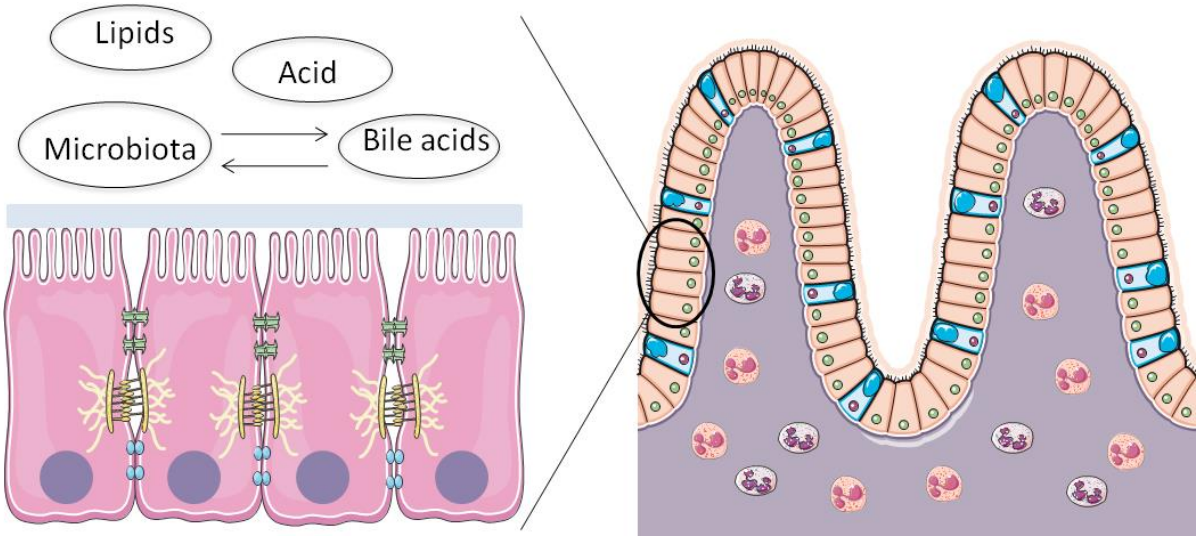


Figure 6.1 Interplay between luminal factors and mucosal defense mechanisms. We hypothesize that, due to the decreased primary to secondary BA ratio, the luminal BA pool has more detergent effects on the mucosal barrier, resulting in impaired duodenal mucosal integrity. An increased mucosal permeability may allow the passage of luminal antigens through the mucosa, evoking immune responses. By nerve-mediated activation by corticotropin-releasing hormone or cholinergic pathways, mucosal mast cells release multiple mediators effecting transcellular and/or paracellular permeability. This may lead to an inappropriate immune activation that in a susceptible host results in mucosal inflammation. During the inflammation, cytokines are released and activate eosinophils. During eosinophil degranulation, amongst others, nerve growth factor and major basic proteins are released and may provoke visceral hypersensitivity and altered gastric motor control, finally resulting in dyspeptic symptom generation.

6.2 Is ursodeoxycholic acid a promising treatment for patients with functional dyspepsia?

We performed the first UDCA trial in patients with FD and we recorded no evidence that UDCA has a protective effect in the pathophysiology of FD in this pilot study. Furthermore, increasing concentrations of UDCA trend towards an increase in the metabolic activity of cultured caco-2 cells in our *in vitro* model. Finally, GUDCA seems to enhance the epithelial resistance in duodenal biopsies from patients with FD in an Ussing chamber experiment.

Considering our results, a complex ratio of primary and secondary BA, in which GUDCA and TUDCA are involved, correlate with duodenal permeability in patients with FD. This correlation is opposite to what we expected of UDCA conjugates, as it seems that increased concentrations of GUDCA and TUDCA are associated with increased duodenal mucosal permeability. Moreover, duodenal mucosal epithelial resistance was decreased in patients with FD, suggesting impaired duodenal barrier function. Exposure of duodenal biopsies to GUDCA slightly altered the TEER, resulting in similar TEER

values between patient and HV biopsies. Contrary to our expectations, less bacterial passage was observed in biopsies from patients with FD compared to HV and GUDCA exposure increased bacterial passage through patient biopsies, resulting in a similar bacterial passage as through HV biopsies. Furthermore, the results of the caco-2 cell experiment suggest that the increased presence of UDCA and UDCA conjugates has mild protective effects on membrane integrity *in vitro*. This may be due to the observation that UDCA protects by increasing the rate of metabolic activity and/or cell proliferation of the cells. Finally, In a heterogenous pool of eight patients with FD, UDCA had no significant effect on duodenal permeability, immune cell count, BA receptor expression, IGP and symptom occurrence. However, the effects of UDCA on those parameters vary strongly interindividually and do not link with a specific FD subtype (PDS, EPS and PDS/EPS overlap group). Also, during UDCA intake, a correlation was found between BA and duodenal permeability and gastric discomfort in patients with FD.

Currently, researchers are investigating the use of UDCA as therapy for intestinal inflammatory disorders including IBD, ileitis, and colon cancer.[20] UDCA has cytoprotective effects by, amongst others, inhibiting apoptosis, stabilizing plasma membranes, reducing BA cytotoxicity, acting as an antioxidant and attenuating the elevation in inflammatory cytokines and intestinal villus damage.[20-21] In a study from Van den Bossche et al, oral administration of UDCA in a dextran sodium sulfate (DSS)-induced colitis mice model decreased colitis severity and normalized the ratio of Firmicutes to Bacteroidetes.[22] It was shown by Mroz et al that UDCA promotes wound healing and may be an effective treatment in IBD by modulating the intestinal barrier function.[23] According to data from Kim et al, UDCA may be used as a protective agent against chemotherapy-associated intestinal mucositis.[21] 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.[24]

Considering our results, there may still be a rationale for BA targeting interventions in patients with FD with severely impaired mucosal integrity. However, UDCA may not be the most appropriate therapy for FD, as UDCA is only metabolized into LCA by gut microbiota in the colon, resulting in a not fully expression of its protective effects in the duodenum.

6.3 Future prospects

According to chapter 3, further research is necessary to establish to which extent a decreased duodenal BA concentration and primary to secondary BA ratio may play a role in the pathophysiology of FD. Powered treatment trials with administration of exogenous BA (ursodeoxycholic acid, obeticholic acid) may be required to fully address this issue. In addition, it remains uncertain whether these changes in total BA and VDR expression are a cause of the pathophysiological mechanisms in FD or reflect a consequence of the change in diet in patients who often suffer from postprandial dyspeptic symptoms. Hence, a causal relationship needs to be established in future experiments. Furthermore, vitamin D blood levels and the marker for BA synthesis need to be measured in patients in order to validate the present results and to determine which role vitamin D and VDR plays in the pathophysiology of FD.

Discussing the bacterial translocation study, the reported effects of GUDCA on duodenal permeability and *E. Coli* passage ask for more intensive research in the effects of individual as well as combined BA on duodenal permeability and bacterial passage and their role in the pathophysiology of FD. For future research, it is essential to not only use multiple BA in this experimental setup, but also living, duodenum and/or FD specific bacteria. Also, re-evaluation of the correlation between BA and intestinal permeability in FD should be considered in a larger sample size.

Considering the UDCA trial, it is advised to perform a new trial with a larger sample size, a longer medication period and a homogenous patient pool characterized by a decreased duodenal mucosal resistance. This trial will be necessary to correctly understand the underlying mechanisms of UDCA in the pathophysiology of FD and will decide whether UDCA can be used in the future as a treatment or part of a treatment for a subgroup of patients with FD. To clearly understand the role of microbiota in FD, it is advised to evaluate and compare the duodenal mucosal microbiota between HV and patients with FD in a study with a large sample size using an aseptic biopsy device, such as the BABD to avoid cross-contamination.

6.4 General conclusion

We can conclude that patients with FD displayed decreased duodenal BA concentrations during fasting and an increased duodenal vitamin D receptor expression. In addition, the hydrophilic BA GUDCA ameliorates the epithelial resistance of duodenal patient biopsies and it seems that duodenal BT does not play a role in the pathophysiology of FD. Finally, we report that a powered BA pool directed trial should be performed to fully understand the contribution of BA in the pathophysiology of FD.

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Chapter 7

Summary

7 Summary

Functional dyspepsia (FD) is a complex disorder of the upper gastrointestinal tract in which a variety of pathophysiological mechanisms such as impaired duodenal barrier function and low-grade inflammation are involved. However, the cause and mechanism underlying FD is still unclear and the factor(s) causing impaired duodenal barrier function and low-grade inflammation has not been identified. Likely candidates are genetic factors, stress and duodenal luminal factors, including bile acids (BA). Considering the limited availability of efficient treatment and the high health care cost, it is essential to find an efficient and target-oriented treatment for patients with FD.

We hypothesized that increased duodenal BA levels and/or a different BA composition leads to impaired duodenal mucosal integrity in FD, allowing luminal substances to pass through the mucosa and resulting in low-grade immune responses. This could provoke visceral hypersensitivity and altered gastric motor control, finally resulting in dyspeptic symptom generation. On the other hand, increased duodenal permeability could lead to an increased passage of BA, resulting in visceral hypersensitivity and/or altered gastric motor control directly or indirectly via low-grade inflammation. To accept or reject this hypothesis, we characterized the duodenal BA composition and receptor expression in FD, we analyzed the relationship between BA, bacterial translocation (BT) and duodenal mucosal integrity in FD, and we evaluated the effect of UDCA treatment on duodenal permeability, low-grade inflammation, the gut microbiome and meal related sensory motor function in FD.

Gastroduodenoscopy was performed to obtain duodenal biopsies from healthy volunteers (HV) and patients with FD to measure duodenal BA receptor expression with western blot. The remaining duodenal biopsies were mounted in the Ussing chambers to measure transepithelial electrical resistance (TEER) and bacterial passage using fluorescein conjugated *Escherichia coli* and glycoursodeoxycholic acid (GUDCA). After the gastroduodenoscopy, a catheter was positioned in the duodenum and duodenal fluid aspirates were collected at fixed time points during fasted and fed state conditions and BA composition and concentration analysis was performed by liquid chromatography-mass spectrometry/mass spectrometry. In addition, caco-2 cells were exposed to ursodeoxycholic acid (UDCA) with or without exposure to duodenal aspirates from patients with FD or HV to evaluate the effect of UDCA on mucosal integrity. Finally, a double blind, placebo controlled, randomized clinical trial was performed in 8 patients with FD to evaluate the effect of UDCA on duodenal permeability, low-grade inflammation, gut microbiome and meal related sensory motor function.

In chapter 3, we observed that patients with FD displayed decreased duodenal BA concentrations during fasting and an increased duodenal mucosal vitamin D receptor expression compared to HV. We also found a decreased primary to secondary BA ratio and some correlations between duodenal BA and symptom pattern in FD. In chapter 4, we showed that the duodenal mucosal TEER from as well as *E. Coli* passage through biopsies of patients with FD were lower compared to HV. With GUDCA exposure, TEER and *E. Coli* passage were similar in patient and HV biopsies. We also reported a correlation between BA and duodenal permeability and no correlation between duodenal permeability and *E. Coli* passage. In chapter 5, we reported increased duodenal GUDCA and tauroursodeoxycholic acid (TUDCA) concentrations in patients with FD after four weeks of oral UDCA intake. However, the patient group displayed a similar TEER, paracellular passage and dyspeptic symptom occurrence during placebo and UDCA intake.

We can conclude that patients with FD displayed decreased duodenal BA concentrations during fasting and an increased duodenal vitamin D receptor expression. In addition, the hydrophilic BA GUDCA ameliorates the epithelial resistance of duodenal patient biopsies and it seems that duodenal BT does not play a role in the pathophysiology of FD. Finally, we report that a powered BA pool directed trial should be performed to fully understand the contribution of BA in the pathophysiology of FD.

Chapter 8

Samenvatting

8 Samenvatting

Functionele dyspepsie (FD) is een functionele gastrointestinale aandoening, waarbij verschillende pathofysiologische mechanismen, zoals verhoogde duodenale doorlaatbaarheid en laaggradige duodenale inflammatie, betrokken zijn. De oorzaak en het mechanisme van FD is nog onduidelijk en de factor(en) die verhoogde duodenale doorlaatbaarheid en laaggradige duodenale inflammatie veroorzaken, is/zijn nog niet geïdentificeerd. Kandidaten hiervoor zijn genetische factoren, stress en duodenale luminale factoren zoals galzouten. Omwille van de beperkte beschikbaarheid van efficiënte behandelingen voor FD en omwille van de hoge gezondheidskost, is het essentieel om een efficiënte behandeling te vinden voor patiënten met FD.

Wij veronderstelden dat verhoogde duodenale galzoutconcentraties en/of een verschillende galzoutsamenstelling in patiënten met FD leidt tot verhoogde duodenale doorlaatbaarheid. Hierdoor kunnen luminale substanties doorheen de mucosa passeren, wat resulteert in laaggradige inflammatie. Dit kan dan weer viscerale hypersensitiviteit en een veranderde maag motoriek uitlokken, resulterend in het ontstaan van dyspeptische symptomen. Anderzijds kan verhoogde duodenale doorlaatbaarheid leiden tot een verhoogde passage van galzouten, resulterend in viscerale hypersensitiviteit en/of een veranderde maag motoriek direct of indirect via laaggradige duodenale inflammatie. Om deze hypothese te aanvaarden of te verwerpen, evalueerden wij de duodenale galzoutsamenstelling en receptorexpressie in FD, analyseerden wij de relatie tussen galzouten, bacteriele translocatie en de duodenale mucosale integriteit in FD, en wij evalueerden het effect van ursodeoxycholzuur behandeling op duodenale doorlaatbaarheid, laaggradige duodenale inflammatie, de intestinale microbioom en de maaltijd gerelateerde sensorische motor functie in FD.

Een gastroscopie werd uitgevoerd om duodenale biopten te verkrijgen van patiënten en gezonde vrijwilligers om de duodenale galzoutreceptorexpressie te evalueren met western blot. De overige biopten werden gemonteerd in de Ussing kamers om transepitheliale electrische weerstand en bacteriële doorlaatbaarheid te meten. Hiervoor werd gebruik gemaakt van fluoresceine geconjugeerde *Escherichia Coli* en glycoursodeoxycholzuur. Na de gastroscopie werd een katheter in het duodenum geplaatst en duodenaal vocht werd geaspireerd en verzameld op vaste tijdstippen tijdens nuchtere en gevoede toestand. De galzoutsamenstelling en –concentratie werd geanalyseerd door middel van een vloeistof chromatografie-massaspectrometrie/massaspectrometrie. Daarnaast werden caco-2 cellen blootgesteld aan ursodeoxycholzuur met of zonder blootstelling aan duodenale aspiraten van patiënten met FD of gezonde vrijwilligers om het effect van ursodeoxycholzuur op mucosale integriteit te evalueren. Ten slotte werd een dubbel blinde, placebo gecontroleerde,

gerandomisserde klinische studie uitgevoerd in 8 patiënten met FD om het effect van ursodeoxycholzuur op duodenale doorlaatbaarheid, laaggradige inflammatie, de intestinale microbioom en de maaltijd gerelateerde sensorische motor functie te meten.

In hoofdstuk 3 ondervonden we dat patiënten met FD verlaagde duodenale galzoutconcentraties tijdens de gevaste toestand en verhoogde duodenale mucosale vitamine D receptor expressie vertonen in vergelijking met gezonde vrijwilligers. We vonden ook een verlaagde primaire tot secundaire galzouten ratio en enkele correlaties tussen duodenale galzouten en symptomen in FD. In hoofdstuk 4 toonden we aan dat de duodenale mucosale epitheliale weerstand van en de *E. Coli* passage doorheen biopten van patiënten met FD lager waren dan in biopten van gezonde vrijwilligers. Met blootstelling aan glycoursodeoxycholzuur waren de duodenale mucosale epitheliale weerstand van en de *E. Coli* passage doorheen de biopten van patiënten met FD gelijkaardig als in de biopten van gezonde vrijwilligers. We rapporteerden ook een correlatie tussen galzouten en duodenale doorlaatbaarheid en geen correlatie tussen duodenale doorlaatbaarheid en *E. Coli* passage. In hoofdstuk 5 rapporteerden we verhoogde duodenale glyco- en tauroursodeoxycholzuur concentraties in patiënten met FD na vier weken orale inname van ursodeoxycholzuur. Verder vertoonden de patiënten een gelijkaardige epitheliale weerstand, paracellulaire passage en symptomen tijdens placebo en ursodeoxycholzuur inname.

We kunnen besluiten dat patiënten met FD verlaagde duodenale galzoutconcentraties vertonen tijdens de gevaste toestand en een verhoogde duodenale vitamine D receptor expressie hebben. Daarnaast verbetert het hydrofiele galzout glycoursodeoxycholzuur de epitheliale weerstand van duodenale biopten van patiënten met FD. Bovendien ziet het er naar uit dat duodenale bacteriële translocatie geen rol speelt in FD. Ten slotte rapporteren we dat een studie gericht op de galzoutenpoel moet worden uitgevoerd met een voldoende steekproefgrootte om de rol van galzouten in de pathofysiologie van FD volledig te begrijpen.

Chapter 9

Acknowledgements, personal contributions and conflict of interest Statement

9 Acknowledgements, personal contributions and conflict of interest Statement

This thesis manuscript is completed with the help and support of many persons, who I want to thank for their contribution. I will mention all contributions per chapter.

Chapter 1: Introduction

This chapter was written by D. Beeckmans and revised by J. Tack.

Chapter 2: Research objectives

The research objectives of this doctoral project were designed by D. Beeckmans, R. Farré, T. Vanuytsel, J. Tack and H. Vanheel.

This chapter was written by D. Beeckmans and revised by J. Tack.

Chapter 3: Altered duodenal bile acid concentration and receptor expression in functional dyspepsia

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Beeckmans D, Vanuytsel T, Farré R, Tack J and Vanheel H conceived and designed the study protocol. Tack J and Vanheel H acquired funding. Riethorst D, Augustijns P and Tack J provided technical services and materials. Beeckmans D, Vanuytsel T and Tack J recruited participants. Beeckmans D and Riethorst D performed the experiments and analyzed data. Beeckmans D interpreted results of experiments, wrote the manuscript and prepared the figures and tables. Riethorst D, Augustijns P, Vanuytsel T, Farré R, Tack J and Vanheel H revised and edited the manuscript. Beeckmans D finalized the manuscript.

In addition, the authors would like to thank the nursing team of endoscopy for their collaboration.

Chapter 4: Relationship between bile acids, bacterial translocation and duodenal mucosal integrity in functional dyspepsia

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tables. Vanuytsel T and Tack J revised and edited the manuscript. Beeckmans D finalized the manuscript.

In addition, the authors would like to thank the nursing team of endoscopy for their collaboration and Toth J for his assistance with ussing chamber experiments.

Chapter 5: Effect of ursodeoxycholic acid on duodenal permeability, low-grade inflammation, gut microbiome and meal related sensory motor function in functional dyspepsia

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Chapter 6: General discussion and future prospects

This chapter was written by D. Beeckmans and revised by J. Tack.

Chapter 7: Summary

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Chapter 8: Samenvatting

This chapter was written by D. Beeckmans and revised by J. Tack.

Chapter 9: Acknowledgements, personal contributions and conflict of interest Statement

This chapter was written by D. Beeckmans.

Conflict of interest

I have no conflicts of interest to declare.

CURRICULUM VITAE

Dorien Beeckmans was born on December 3, 1991 in Soest, Germany. After finishing Latin-Mathematics at Sint-Janscollege in Meldert, she started her training in Biomedical Sciences at the University of Leuven, Belgium. She obtained her Bachelor's Degree in 2012 and her Master's Degree in 2014. In October 2014, she started her doctoral training at the Translational Research Center for Gastrointestinal Disorders (TARGID), University of Leuven, Belgium under the supervision of Prof. Jan Tack, Dr. Hanne Vanheel and Prof. Tim Vanuytsel. Her scientific work has contributed to several abstracts and a peer-reviewed publication.

LIST OF PUBLICATIONS

Publications

Dorien Beeckmans, Danny Riethorst, Patrick Augustijns,Tim Vanuytsel,Ricard Farré, Jan Tack, Hanne Vanheel. Altered duodenal bile salt concentration and receptor expression in functional dyspepsia. Accepted by the United European Gastroenterology Journal on the 31st of July 2018.

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Awaiting Publications

Beeckmans Dorien, Farré Ricard, Riethorst Danny, Keita Åsa V, Augustijns Patrick, Söderholm Johan D, Vanuytsel Tim, Tack Jan,Vanheel Hanne. Relationship between bile salts, bacterial translocation and duodenal mucosal integrity in functional dyspepsia.

Dorien Beeckmans, Linda Holler, Ricard Farré, Danny Riethorst, Maria Vicario, Patrick Augustijns, Jeroen Raes, Jan Tack. Pilot study: Effect of ursodeoxycholic acid on duodenal permeability, low-grade inflammation, gut microbiome and meal related sensory motor function in patients with FD

Dorien Beeckmans, Jan Tack. Revisiting the duodenum in the pathophysiology of functional dyspepsia: duodenal mucosa and luminal content.

Meeting abstracts

Giancola F, Volta U, Repossi R, Latorre R,Beeckmans D, Caio G,Vanuytsel T, StanghelliniV, Tack J, De Giorgio R. Neuroimmune mechanisms in symptom generation in patients with non-celiac gluten / wheat sensitivity. Abstract accepted for the Federation of Neurogastroenterology and Motility (FNM) Meeting, Amsterdam, the Netherlands, August 2018.

Dorien Beeckmans, Ricard Farré, ÅsaV Keita, Johan D Söderholm, Tim Vanuytsel, Jan Tack, Hanne Vanheel. The effect of bile salts on epithelial integrity and bacterial uptake in functional dyspepsia. Abstract accepted for poster presentation at the Digestive Disease Week (DDW), Washington DC, USA, June 2018.

Hanne Vanheel, Dorien Beeckmans, Maria Vicario, Jan Tack, Ricard Farré, Tim Vanuytsel. Duodenal acidification induces impaired duodenal integrity and activation of the duodenogastric reflex, independent from mast cell activation. Abstract accepted for oral presentation at the FNM Meeting, Cork, Ireland, August 2017.

Dorien Beeckmans, Ricard Farré, Åsa Keita, Johan Soderhölm, Jan Tack, Hanne Vanheel. The effect of glycoursodeoxycholic acid on epithelial integrity and bacterial uptake in duodenal biopsies of patients with functional dyspepsia and healthy volunteers. Abstract accepted for poster presentation at the FNM Meeting, Cork, Ireland, August 2017.

D Beeckmans, D Riethorst, R Farré, P Augustijns, J Tack,H Vanheel. Association between luminal bile salt content and duodenal mucosal integrity in functional dyspepsia. Abstract accepted for oral presentation at DDW, Chicago, USA, May 2017.

D Beeckmans, D Riethorst, R Farré, P Augustijns, J Tack,H Vanheel. Association between luminal bile salt content and duodenal mucosal integrity in healthy volunteers. Abstract accepted for oral presentation at the United European Gastroenterology Week (UEGW), Vienna, Austria, October 2016.

D Beeckmans, D Riethorst, R Farré, P Augustijns, J Tack, H Vanheel. Pilot study: Role of bile acids in generation of impaired duodenal mucosal integrity. Abstract accepted for poster presentation at the FNM Meeting, San Francisco, USA, August 2016.

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ROLE OF BILE ACIDS IN FUNCTIONAL DYSPEPSIA

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