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MECHANISMS OF SYMPTOM GENERATION AND SYMPTOM PERCEPTION IN REFRACTORY GASTRO-ESOPHAGEAL REFLUX DISEASE

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Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
δENaC	delta subunit of the epithelial sodium channel
5-HIAA	5-hydroxyindoleacetic acid
AET	acid exposure time
AIM	automated-impedance manometry
ASIC	acid-sensitive ion channels
ATD	acute tryptophan depletion
BBB	blood brain barrier
b.i.d	bis in die (twice a day)
BMI	body mass index
CNS	central nervous system
CRH	corticotropin-releasing hormone
DCI	distal contractile integral
DIS	dilated intercellular spaces
ECG	electrocardiogram
EE	erosive esophagitis
EES	extra-esophageal symptoms
EGJ	esophagogastric junction
ELISA	enzyme-linked immunosorbent assay
FH	functional heartburn
FSSG	frequency scale for symptoms of GERD
$GABA_B$	γ-amino-n-butyric acid
GER	gastro-esophageal reflux
GERD	gastro-esophageal reflux disease
GC-MS	gas chromatography - mass spectrometry
GI	gastro-intestinal
HCl	Hydrochloric acid
HH	hiatal hernia
HPA	hypothalamic-pituitary-adrenal axis
HRM	high resolution manometry

HRiM	high resolution impedance manometry
HV	healthy volunteer
IBP	intrabolus pressure
IBS	irritable bowel syndrome
IEM	ineffective esophageal motility
IR	impedance ratio
IRP	integrated relaxation pressure
IV	intravenous
LES	lower esophageal sphincter
LNAA	large neutral amino acid
mGluR5	metabotropic glutamate receptor 5
MII-pH	multichannel intraluminal impedance-pH
MMS	medical measurement systems
NaCl	sodium-chloride
NERD	non-erosive reflux disease
NSAID	non-steroidal anti-inflammatory drugs
PANAS	positive and negative affect schedule
PAR-2	protease-activated receptor 2
PCR	polymerase chain reaction
PET	positron emission tomography
PFA	pressure flow analysis
PFI	pressure flow index
PPI	proton pump inhibitor
PPT	pain perception threshold
PTT	pain tolerance threshold
rGERD	refractory gastro-esophageal reflux disease
SAP	symptom association probability
SC	subcutaneous
SI	symptom index
SSRI	selective serotonin re-uptake inhibitor
STAI	state-trait anxiety inventory
UES	upper esophageal sphincter
TEER	transepithelial electrical resistance

TEM	transmission electron microscopy
TLESR	transient lower esophageal sphincter relaxation
TRP	tryptophan
TRPV1	transient receptor potential vanilloid receptor type-1

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CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Gastro-esophageal reflux disease

Gastro-esophageal reflux (GER) is the retrograde flow of gastric contents into the esophagus, which is a physiological phenomenon. However, when GER is causing troublesome symptoms, such as heartburn and regurgitation, or lesions, it is referred to as gastro-esophageal reflux disease (GERD) (1). GERD is a frequent condition affecting about 10 to 30% of the adult Western population (2). It may present with a broad spectrum of symptoms, divided into typical or esophageal manifestations of which heartburn and regurgitation are most important and a variety of atypical, extra-esophageal symptoms (EES), such as chronic cough, wheezing and hoarseness (1, 3-6). In addition, increased exposure of the esophageal epithelium to noxious gastric contents, such as acid, bile salts and pepsin, can lead to severe conditions such as erosive esophagitis (EE), peptic strictures, and Barrett's esophagus, which can develop into esophageal adenocarcinoma (7-9).

1.2 Diagnosis of GERD

The presence of GERD can be measured in a number of different ways. First, GERD can be quantified by questioning symptom frequency and symptom severity (10). Several questionnaires can be applied, *e.g.* in 2004 Kusano *et al.* developed the frequency scale for symptoms of GERD (FSSG). This widely used, simplified questionnaire for evaluation of the symptoms of GERD addresses 12 questions ranging from heartburn, a heavy feeling after meals, to burning sensations in the throat (11). The ReQuest questionnaire is another example of a widely used, self-reported questionnaire which was developed to assess the broad spectrum of GERD symptoms (12). The use of questionnaires has the major advantage that these are relatively easy to apply, non-invasive methods to assess the presence of GERD symptoms. However, the most common drawback of a questionnaire-based strategy is the subjectivity and in many cases also the absence of established cut-off points. Except for the GerdQ questionnaire, which has an established and validated cut-off point of GerdQ ≥9. Therefore, GerdQ is considered to be a useful complementary tool for the diagnosis of GERD. (13).

Ambulatory reflux monitoring is considered the gold standard to quantify GER and the use of esophageal pH monitoring is widely available. In addition, already more than a decade ago

impedance measurements have been added to conventional pH monitoring. This combined multichannel intraluminal impedance-pH monitoring (MII-pH) detects not only acid and non-acid reflux but also provides additional information concerning the composition of the refluxate (liquid, gas, mixed) and the proximal extent of reflux events (14, 15). Especially in patients with refractory GERD symptoms this additional information is of great importance since in this population reflux monitoring is often performed while patients are on acid suppressive medication (16). However, particularly in the case of atypical GERD symptoms, the diagnostic accuracy of MII-pH monitoring appeared to be rather modest (17, 18).

In the past, several groups have tried to determine cut-off values defining pathological reflux. Although they are based on 24 hour pH or MII-pH monitoring, they are not always consistent (19-21). Therefore, very recently, Roman *et al.* published a review article concerning an international consensus for the diagnosis of GERD using ambulatory reflux monitoring. This consensus group suggested international cut-off values for reflux monitoring both 'on' and 'off' acid suppressive therapy (22).

Upper endoscopy is an excellent tool in the evaluation of consequences of reflux, since it has the advantage of providing luminal and objective assessments of the presence of GERD lesions. However, a large proportion of patients with GERD have no macroscopic lesions, and this method does not allow to distinguish microscopic changes in esophageal mucosa that may be the underlying cause of symptoms in some individuals. Bredenoord *et al.* concluded that endoscopy is a test with high specificity but low sensitivity for GERD diagnosis (15). Besides macroscopic investigation of the esophagus by means of endoscopy, histologic assessment to verify the presence of dilated intercellular spaces (DIS) and microscopic esophagitis can also be used to support the diagnosis of GERD (23, 24).

Empirical use of proton pump inhibitors (PPIs), in the form of a short trial (mostly 1 to 4 weeks) of PPI treatment, can be useful since resolution of symptoms may render additional diagnostic testing unnecessary (15, 25). On the other hand, a favorable response to high dosages of PPIs is not specific and does not confidently diagnose GERD (25).

Finally, investigating the presence of (duodeno-) gastric contents, such as pepsin or bile acids, in the saliva of patients with symptoms suggestive of GERD, may also identify GERD (26). However, Hayat *et al.* demonstrated a substantial overlap between healthy controls and GERD

patients when measuring presence of pepsin in saliva. The sensitivity and specificity of salivary pepsin detection are not superior but rather similar to those achieved by other methods for GERD diagnosis, with the major advantage that it is a non-invasive and inexpensive diagnostic tool (26).

1.3 Pathophysiology of GERD

GERD is a complex and multifactorial disease with a wide range of possible clinical presentations that remain incompletely understood (27). The factors that have been suggested to contribute to the pathogenesis of GERD are shown in Figure 1.1 and will be further discussed in this section.

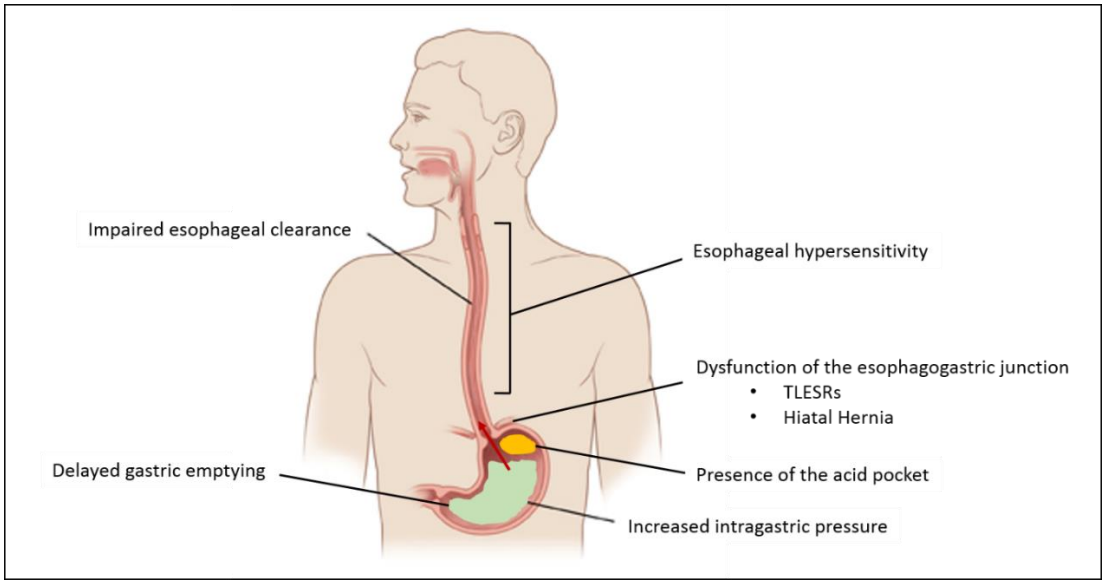

Figure 1.1 Pathophysiology of gastro-esophageal reflux disease. Abbreviations: TLESRs= transient lower esophageal sphincter relaxations. Based on Bredenoord *et al.* 2013 and Boeckxstaens *et al.* 2014 (7, 15).

1.3.1 Dysfunction of the esophagogastric junction

The most important anti-reflux mechanism is the esophagogastric junction (EGJ) which consists of the lower esophageal sphincter (LES), the angle of His and the muscle fibers of the crural diaphragm. The LES, which consists of circular smooth muscle fibers located at the distal end of the esophagus, relaxes during each swallow to allow a bolus to pass into the stomach. Together with the LES, the crural diaphragm constitute the intrinsic and extrinsic sphincters, respectively. These two sphincters are anatomically superimposed and are anchored to each other by the phrenoesophageal ligament (28).

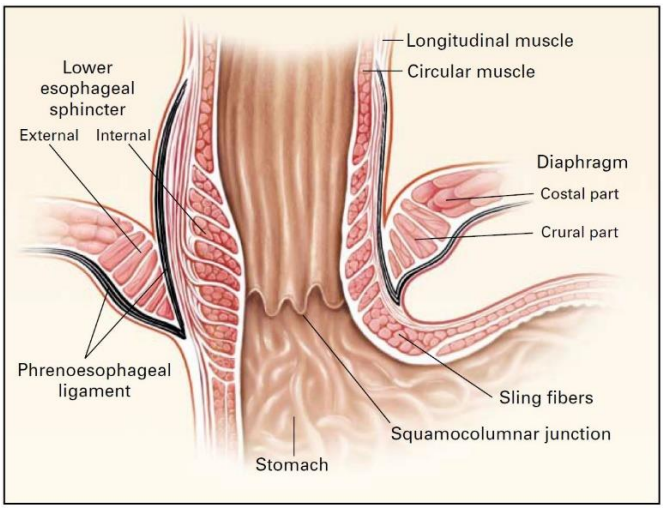

Figure 1.2 Schematic overview of the esophagogastric junction consisting of the lower esophageal sphincter, the angle of His and the crural diaphragm. Reproduced with permission from Mittal *et al.*, (28) Copyright Massachusetts Medical Society.

The LES plays a crucial role in the frequency of reflux events and the severity of GERD. The normal resting tone of the LES ranges from 10-35 mmHg relative to intragastric pressure. The LES maintains a high pressure zone by tonic contraction mainly through neurogenic mechanisms mediated by cholinergic nerves. During the day, there are considerable variations in basal LES pressure; it is lowest after a meal and highest during sleep. Furthermore, LES pressure can also be altered by a number of circulating peptides and hormones or food, particularly fat. When increases in intragastric pressure overcome a hypotensive LES, reflux can occur (15, 29). However, it has been postulated that transient relaxations of the LES (TLESRs) are the principal mechanism that leads to reflux events both in healthy subjects and in patients with GERD (30). A TLESR is a period of simultaneous relaxation of the LES and crural diaphragm, independent of swallowing. It is a neural reflex that is mediated through afferents of the vagus nerve in the cardia of the stomach and is triggered by various stimuli, of which gastric distention is the most frequent (7, 30). Furthermore, pharyngeal stimulation, body postures and meals high in fat are also suggested to influence the occurrence of TLESRs (28). Although the frequency of TLESRs in patients with GERD is not different from that in healthy subjects (31), the occurrence of reflux during a TLESR is more likely in patients with GERD compared to healthy subjects (32).

As mentioned above, under normal circumstances, a synergistic high pressure zone is created by the distal part of the LES located in the abdomen and the crural diaphragm. However, in the presence of a hiatal hernia a part of the stomach migrates more proximal into the thoracic

cavity, separating these two high pressure zones (33). When separated, pressures of both sphincter zones become much weaker (34). The cause of a hiatal hernia is not completely clarified, however, increasing body weight and abdominal obesity are suggested to be risk factors (27).

There are a number of mechanisms by which a hiatal hernia may promote reflux. First, it impairs clearance of acid from the esophagus. During swallowing, the LES relaxes and the distal esophagus is unprotected against gastric acid until the esophageal contractile wave arrives at the distal esophagus. In addition, in case of a hiatal hernia, gastric contents can be trapped in the hiatal sac which may function as a reservoir from which gastric fluids can flow backwards into the esophagus after swallowing or during periods of low LES pressure (28, 33). It has been shown that the presence and size of a hiatal hernia is associated with more severe erosive esophagitis (EE) and Barrett's esophagus due to a prolonged acid exposure time and prolonged acid clearance time (35).

1.3.2 Impaired esophageal clearance

Esophageal peristalsis, triggered by mechanoreceptors in the esophageal lumen, is the main mechanism for clearance of refluxed gastric contents. In addition, salivary bicarbonate contributes to acid clearance due to its neutralizing effect on acid and thereby normalization of esophageal pH (7). It has been shown that prolonged acid clearance correlates with both the severity of esophagitis and the presence of Barrett's metaplasia (36, 37). An intact peristaltic function of the esophagus is therefore an important defense mechanism against GERD. The combination of multichannel intraluminal impedance and high resolution esophageal manometry is an advanced tool to evaluate bolus transit and esophageal motility in GERD (38). In this regard, the presence of ineffective esophageal motility (IEM) can be related to GERD. IEM has been associated with the occurrence of reflux events and has been shown to be almost equally present in patients with erosive and non-erosive reflux disease (39, 40). Furthermore, it has been shown that esophageal motility abnormalities increase in parallel with the severity of GERD. Therefore, bolus transit abnormalities in severe reflux disease underscore the importance of impaired esophageal function in the development of mucosal injury (38).

1.3.3 Increased intragastric pressure

In order for reflux to occur, a positive pressure gradient is needed between the proximal stomach and the distal esophagus. Certain activities such as coughing or straining, lead to a temporary rise in abdominal pressure thereby increasing the gastro-esophageal pressure gradient (15, 41). A chronically increased abdominal pressure is present in obesity and different studies have shown that this is an important risk factor for the occurrence of GERD symptoms, prolonged esophageal acid exposure, esophagitis and even Barrett's esophagus (42-44).

1.3.4 Acid pocket

In 2001 Fletcher *et al.* demonstrated the presence of a highly acidic, unbuffered layer of gastric juice which is present after a meal. This so called 'acid pocket' is located near the EGJ, on top of the meal in the stomach (45). The acid pocket is present in both healthy subjects and patients with GERD and this has been confirmed using different techniques including high-resolution pH monitoring, Bravo capsule, magnetic resonance imaging, and scintigraphy (46). However, the position and size of the acid pocket is different in GERD patients compared to healthy subjects and these alterations can contribute to the occurrence of acid reflux and symptoms of GERD (7, 47).

1.3.5 Delayed gastric emptying

Although a delayed gastric emptying has been found in about a third of GERD patients compared to healthy controls (48), a relation between gastric emptying time and increased reflux or increased esophageal acid exposure could not be demonstrated. This suggests that impairment of gastric emptying as a whole is not an important factor in the pathophysiology of GERD (41). Sifrim *et al.* showed that the rate of gastric emptying might determine the acidity and proximal extent of reflux: the slower the emptying, the higher the pH and proximal extent of the refluxate (48).

1.3.6 Esophageal hypersensitivity

Esophageal hypersensitivity can develop through sensitization of esophageal sensory nerves following the recurrent presence of (duodeno-)gastric contents (49) or indirectly via the production of pro-inflammatory mediators *e.g.* prostaglandin E2 (50, 51). Several factors, which are discussed more in detail below, are proposed to increase or influence esophageal sensitivity such as impaired epithelial barrier function, central sensitization and upregulation

of acid sensitive receptors (52). Hypersensitivity to acid has been described both in patients with EE and in those with macroscopically normal esophageal mucosa. In a subgroup of patients, heartburn symptoms are reported while esophageal acid exposure is within the normal range (7, 15, 52, 53). These patients are referred to as having “reflux hypersensitivity” (see below).

1.4 Refractory GERD symptoms

The association between symptoms of GERD and esophageal lesions is well established. Acid suppressive therapy, especially treatment with PPIs, is highly effective in healing esophagitis but unfortunately it is less efficacious in providing symptom control: 10 to 40% of patients continue to experience reflux symptoms despite optimized PPI therapy (54-56). In 2008, Bredenoord *et al.* suggested to use the term refractory GERD (rGERD) for the condition in which symptoms and/or mucosal lesions, caused by reflux of gastric contents, do not fully disappear or are not responding to a high dose of PPI treatment which implies a double dose of PPI (b.i.d) during a treatment period of at least 12 weeks (57).

The majority of the GERD population (up to 60%) does not show any esophageal lesions at routine upper endoscopy and are therefore referred to as having non-erosive reflux disease (NERD), this is even more the case for the majority of rGERD patients, where PPIs usually have healed esophageal lesions.

In a first, large group of rGERD patients, ongoing pathological weakly acidic reflux despite PPI therapy has been implicated in the pathogenesis of persisting GERD symptoms (‘true’ NERD) (8, 58). In a second group of patients, esophageal hypersensitivity to physiological levels of reflux is thought to be present and generate symptoms. Based on a positive association between occurrence of symptoms and physiological reflux events, these patients are considered to have reflux hypersensitivity. Finally, patients where no association is found between symptom occurrence and reflux events during 24 hour MII-pH monitoring, are referred to as having functional heartburn (FH) (Figure 1.3) (1).

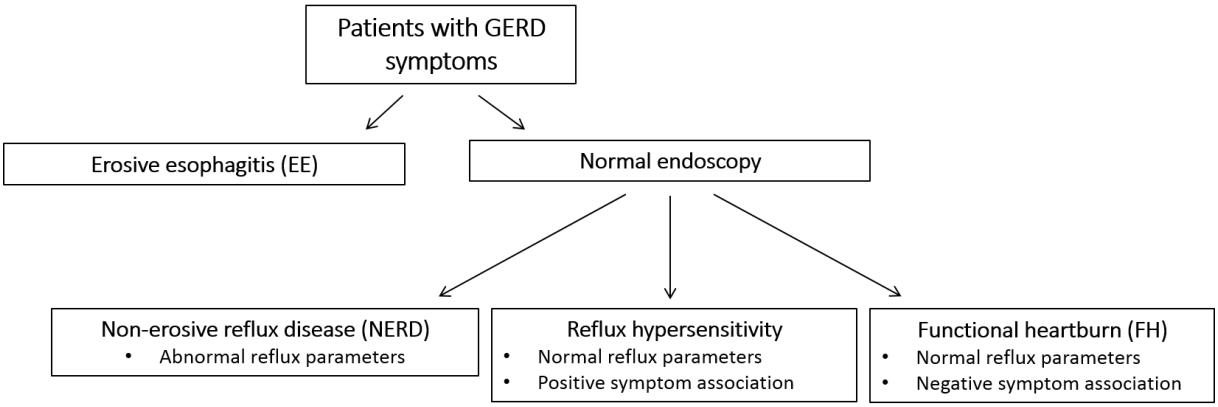

Figure 1.3 Classification of patients with typical GERD symptoms. Based on Rome IV, Aziz *et al.*, 2016 (59).

1.5 Mechanisms underlying refractory GERD symptoms

In literature, a range of underlying mechanisms to explain PPI-resistant GERD symptoms have already been described. These include poor compliance with PPI or insufficient duration of the therapy, persistent volume reflux, insufficient acid suppression or ongoing weakly acidic and non-acid reflux (57, 60). In some cases an alternative diagnosis other than GERD is more likely to be the cause of a patient's symptoms. Functional dyspepsia, rumination syndrome, aerophagia, achalasia and eosinophilic esophagitis are examples of disorders that might be mistaken for GERD (57). Herregods *et al.* demonstrated that approximately 30% of their patient cohort referred with rGERD symptoms suffer from disorders other than GERD, predominantly FH (61). In this section we will discuss some of the mechanisms that are postulated to play a role in rGERD symptoms.

1.5.1 Esophageal sensitivity and failure of anti-nociceptive pathways

Increased visceral sensitivity is a hallmark of many GI disorders which may be caused by excessive sensory transmission from the viscera to the brain (peripheral sensitization), disturbed central processing (central sensitization) or both (62-64). Esophageal hypersensitivity is thought to contribute to symptom generation in patients with 'true' NERD, in those with reflux hypersensitivity and in patients suffering from FH (16, 65). Already more than a decade ago, Trimble *et al.* showed that patients with esophageal acid exposure within the physiological range but with a close correlation between their symptoms and individual reflux episodes, have lower thresholds both for initial perception of and for discomfort during esophageal balloon distention, compared to healthy controls (66). Using a multimodal esophageal stimulation model in a small cohort of rGERD patients on PPI therapy, our own group was able to show that rGERD patients are hypersensitive to thermal, mechanical and

chemical stimulation of the esophagus compared to healthy controls (67). These preliminary data suggest that not only acid and weakly acidic reflux, but also visceral hypersensitivity plays a role in the generation of reflux symptoms and is suggested as a possible mechanism of rGERD symptoms.

Nociceptive receptors present on esophageal nerve endings, can be triggered by chemical, mechanical and thermal stimuli and transmit this information to the central nervous system (CNS) via afferent spinal nerves (68). Esophageal hypersensitivity may be caused by peripheral or central sensitization to stimuli in the gastrointestinal (GI) tract (7, 68). Altered or upregulated expression of these sensory receptors may represent a mechanism of peripheral sensitization. Receptors of the transient receptor potential (TRP) cation channel family are known to play a pivotal role in the transmission of pain sensations (69). In this regard, the transient receptor potential vanilloid type-1 (TRPV1), found on sensory afferent nerve endings, has been studied most extensively in relation to esophageal acid exposure and symptom perception (70). Animal studies have already shown that TRPV1 plays an important role in mechanosensitivity and acid-induced esophagitis (71, 72). In human studies, an upregulation of the expression of TRPV1 and protease-activated receptor 2 (PAR-2) was reported in esophageal mucosa of NERD patients, and are therefore suggested to play a role in symptom generation (70, 73). Furthermore, a recent study by Kim *et al.* showed that acid-induced inflammation in the esophagus was associated with upregulation of mRNA expression of TRPV1 and PAR-2 in patients with EE and NERD, which might lead to the manifestation of reflux symptoms (74). The expression and distribution of acid-sensitive ion channels (ASIC) such as ASIC1, ASIC2 and ASIC3 in the esophageal epithelium remains to be further elucidated, but these receptors are also likely to be involved in chemosensation and mechanosensation (75, 76). Other sensory receptors such as the delta subunit of the epithelial sodium channel (δENaC) have also been suggested to be located in the esophageal epithelium (77), but their role in rGERD symptoms still needs to be clarified. In addition, further research is needed to establish the exact role of the different anti-nociceptive pathways involved in esophageal pain perception and their influence on esophageal sensitivity.

1.5.2 Impaired esophageal integrity

Nociceptors present in the esophageal mucosa, are separated from luminal substances in the esophagus by a tight barrier of squamous epithelium. Cell-to-cell adhesion structures,

consisting of tight junctions, desmosomes and adherens junctions, are distributed around the cell membranes of esophageal epithelial cells and constitute an efficient barrier preventing passage of harmful gastric contents (Figure 1.4) (78).

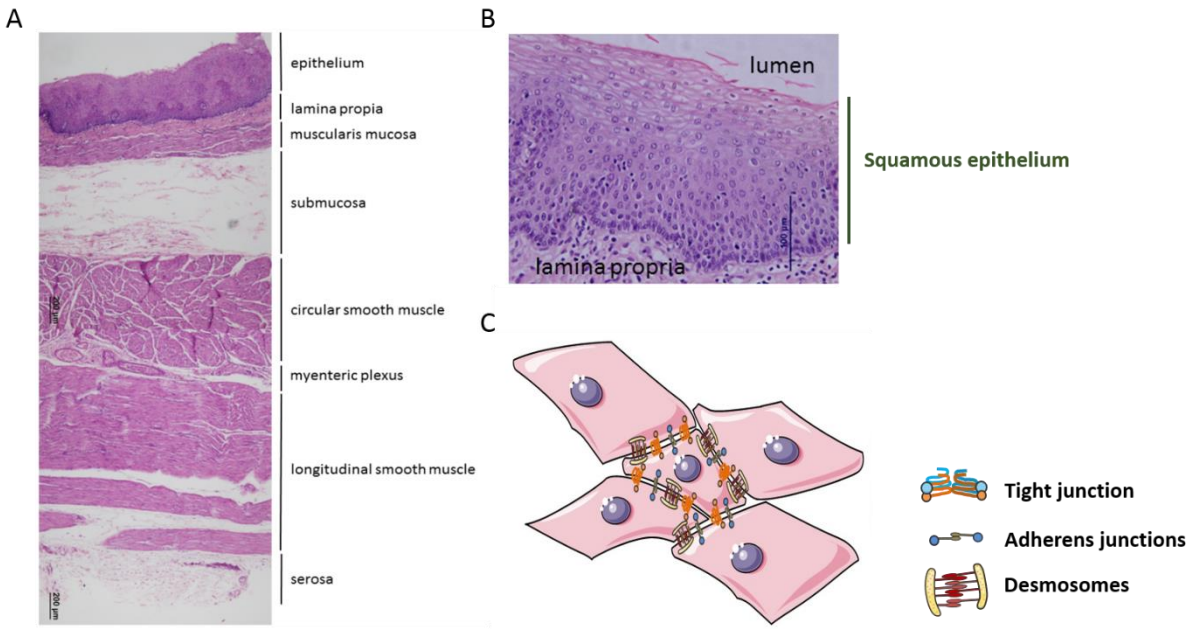

Figure 1.4 Representation of the esophageal epithelium. **A)** Haematoxylin and eosin staining of different layers of the esophageal wall. **B)** Magnified haematoxylin and eosin staining of the squamous esophageal epithelial cell layer. **C)** Schematic representation of the cell-to-cell adhesion structure in the esophageal epithelium. Adapted from Farré, 2013 (78).

Increased exposure to noxious luminal agents can damage the epithelium and thereby lead to an impaired epithelial barrier capacity. Dilated intercellular spaces (DIS) are an accurate morphological marker of impaired epithelial integrity in GERD and are considered to be a typical histopathological finding observed when the epithelium is damaged (Figure 1.5) (62).

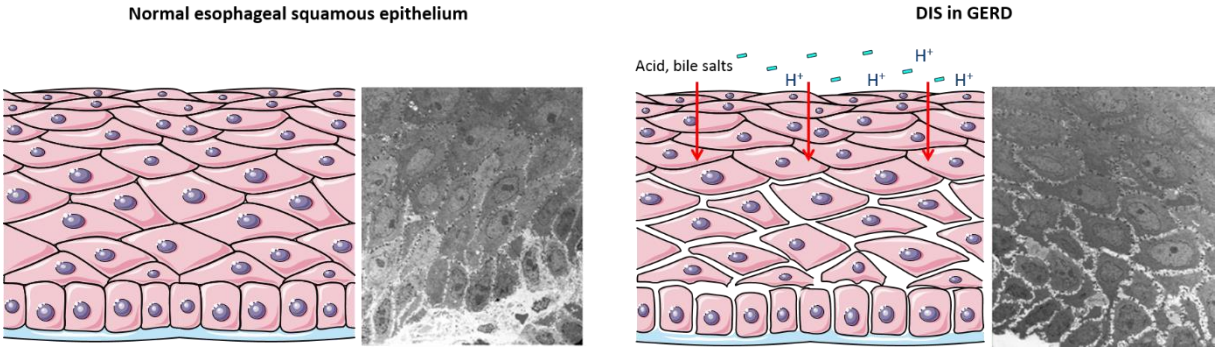

Figure 1.5 Schematic representation and transmission electron microscopic images of normal squamous esophageal epithelium and dilated intercellular spaces present in the epithelium. Adapted from van Malenstein, 2008 (79).

The exact pathway of damage to the intercellular junctions remains unclear and seems to be multifactorial (79, 80). It has been suggested that the proteolytic cleavage (*e.g.* by pepsin) of e-cadherin accounts for the increase in epithelial permeability (81). DIS causes an enhanced access of provocative stimuli such as protons (H^+) or bile acids, which can then trigger the sensory spinal afferent nerves in the lamina propria. Chemosensitive receptors capable to sense these noxious stimuli, are activated and via spinal afferents this signal is transferred to the spinal cord and ultimately to the CNS. Heartburn perception has been associated with presence of an impaired epithelial integrity shown by the presence of DIS in the esophageal epithelium (82, 83).

1.5.3 Psychosocial comorbidities in refractory GERD

Anxiety and depression have a well-established effect on perception of symptoms in functional GI disorders, *e.g.* functional dyspepsia (84, 85). In addition, also in GERD patients it is known that these psychological factors might influence the perception of heartburn, which may reflect a process of central sensitization (86). One candidate trigger for central sensitization are acute or chronic stressful stimuli. Acute auditory stress was able to exacerbate heartburn symptoms both in a group of EE patients as well as NERD patients by increasing the perceptual response to intra-esophageal acid perfusion (87).

In a GERD population referred for work-up, Johnston and colleagues demonstrated that higher anxiety levels were measured most often in patients with FH (88). This was confirmed by Kessing *et al.*, who described that increased anxiety levels were associated with greater severity of retrosternal pain and burning sensation in a cohort of GERD patients (89). In addition, our group also showed an influence of psychological comorbidities and somatization on the reflux-symptom association during 24 hour MII-pH monitoring in GERD patients (90).

Since the severity of GERD symptoms can be affected by increased anxiety levels, the use of anxiolytic agents in a subgroup of GERD patients, mainly patients diagnosed with FH, could have a beneficial outcome. However, further research is needed to determine its effectiveness in clinical practice (89).

1.6 Current treatment options for patients with refractory GERD symptoms

Despite the high prevalence of rGERD, a consensus on the optimal management of this patient population is still lacking. First of all, an extensive patient evaluation is necessary. In this regard

MII-pH monitoring is considered a very useful tool that, if available, can be performed early on in the work-up of patients with rGERD. In most cases, this monitoring is performed while patients are on PPI therapy (b.i.d). However, off PPI monitoring should be used when symptoms are not suspected to be related to reflux or when the patient is not able to use the symptom markers adequately (16, 22).

The first-line treatment option remains PPI therapy since it has a good efficacy in healing EE. However, in patients with persisting heartburn symptoms in spite of optimized PPI therapy, other treatment strategies need to be considered.

One possible alternative treatment are the so-called 'reflux inhibitors'. Since TLESRs are the main mechanism underlying reflux events, they are a potential target for preventing reflux to occur (16). The γ-amino-n-butyric acid ($GABA_B$) receptor agonist baclofen is a potential monotherapy or add-on therapy (91-93). Baclofen reduces GER and its associated symptoms by increasing LES pressure and thereby reducing the occurrence of TLESRs (91). Arbaclofen placarbil, which is a prodrug of baclofen, was designed to have a more favorable adverse effect profile than baclofen. However, a large trial in 460 rGERD patients could not demonstrate significant benefit of this drug as an add-on therapy, therefore discontinuing its further development (94). Besides baclofen, other $GABA_B$ receptor agonists have been investigated. In small clinical trials, lesogaberan, mainly acting on peripheral $GABA_B$ receptors, seemed a promising alternative for baclofen. Unfortunately, in a randomized phase II trial there was no significant improvement in overall symptoms compared to placebo (95). Finally, the metabotropic glutamate receptor 5 (mGluR5) has also been suggested to be involved in the control of TLESRs (96), therefore drugs inhibiting these receptors were thought to be a good candidate in the treatment of rGERD. However, due to potential adverse events and the limited efficacy, none of the mGlur5 inhibitors went into further drug development.

In some cases, prokinetic agents can be considered as an add-on treatment for rGERD since these drugs accelerate gastric emptying, have beneficial effects on LES pressure and enhance esophageal clearance (16). A number of clinical trials have been conducted to investigate the efficacy of prokinetic drugs such as cisapride, mosapride and tegaserod in the treatment of GERD (16, 97). However, to date there is no clear indication that these agents are effective in improving reflux parameters and symptom control (16).

Anti-reflux surgery, a procedure where the anti-reflux barrier is restored, can be an option in a subgroup of patients with rGERD symptoms (98). There are several types of anti-reflux procedures that differ from each other according to the type of fundoplication that is applied. Examples comprise amongst others, the Nissen fundoplication (360°, posterior fundoplication), Toupet fundoplication (270°, posterior fundoplication) and the Belsey-Mark IV procedure (270°, anterolateral fundoplication). The laparoscopic Nissen fundoplication has become the gold standard and is the most commonly performed type of anti-reflux surgery since it is minimally invasive, highly effective and safe (99, 100). The most frequent (though often transient) postoperative complications include dysphagia, early satiation, postprandial fullness, nausea and inability to belch (100-102). Since these complications are likely to occur, the option to treat rGERD patients with anti-reflux surgery should be considered carefully and an adequate patient selection is necessary. In order to select rGERD patients for anti-reflux surgery, an extensive symptom evaluation is warranted. Furthermore, 24 hour MII-pH monitoring off PPI therapy should be performed to select patients that could be eligible for anti-reflux surgery. Pathological esophageal acid exposure is a well-defined selection criterion for anti-reflux surgery, and predicts a more favorable outcome (98). On the contrary, poor response to PPI treatment is a risk factor for unfavorable clinical outcome (16, 103). Trials reporting on the outcome after anti-reflux surgery, mostly Nissen fundoplication, in rGERD are not always consistent: some groups report a favorable symptomatic outcome in patients with a poor PPI response (104-106), while others found a higher persisting symptom burden in patients with refractory GERD in comparison with patients with complete PPI response (103).

Besides the conventional and rather invasive anti-reflux surgery techniques, the development of novel, minimally invasive endoscopic procedures to restore the anti-reflux barrier is being investigated. Radiofrequency ablation therapy (Stretta, Mederi Therapeutics, USA) and the Eso-phyX (EndoGastric Solutions) were introduced more than 10 years ago, however the lack of randomized controlled data in rGERD patients makes it difficult to determine the efficacy of these treatment approaches (107). The magnetic sphincter augmentation device (LINX Reflux Management System, Shoreview, MN, USA) consisting of magnetic beads placed around the EGJ, is intended to augment EGJ pressure thereby preventing reflux to occur while maintaining normal bolus passage and preserving the ability to vomit. However, there is a lack of randomized data currently comparing the LINX procedure to Nissen fundoplication in rGERD

(108). Finally, electrical stimulation of the LES by the EndoStim system device (EndoStim, St-Louis, MO, USA), has been suggested to increase the LES resting pressure thereby controlling reflux events (109). Clinical trials using this device are still ongoing and therefore long-term results to compare the EndoStim with other anti-reflux approaches are currently lacking (108).

Add-on therapies such as therapy with alginates, may be effective in relieving typical reflux symptoms not responding to PPI therapy. Alginates decrease GER by forming a pH-neutral raft localized near the EGJ, at the site of the postprandial acid pocket on top of the gastric content (16, 110). Several trials show that an alginate–antacid combination was superior in reducing the number of acid reflux episodes, but not non-acid reflux episodes (110), or can offer additional decrease in the burden of reflux symptoms (111). Only recently Savarino *et al.* published an article demonstrating the beneficial effects of mucosal protection with Esoxx (a hyaluronic acid-chondroitin sulphate based bioadhesive formulation) in combination with acid suppressive therapy. Mucosal protection by Esoxx in combination with acid suppression accomplished with PPI treatment improved symptoms and quality of life scores in NERD patients (112).

Another therapeutic strategy, focusing on drugs designed to target acid-sensitive receptors was explored by Krarup and colleagues and sounded very promising. However, two clinical trials with a TRPV1 antagonist failed to alter esophageal sensitivity in healthy controls except for minor changes in thermal sensitivity. In a follow-up study, no analgesic effect of TRPV1 antagonism on esophageal pain could be measured in NERD patients with a partial response to PPI treatment (113, 114).

As mentioned earlier, when no association can be found between symptoms of GERD and the occurrence of reflux assessed by MII-pH monitoring, patients are considered to have FH. Since comorbidities such as anxiety disorders and depression seem to be more prevalent in patients with functional GI disorders (89), add-on treatment with psychotropic agents can be considered (16). In addition, because of their effect on pain-processing pathways in the CNS, selective serotonin re-uptake inhibitors (SSRIs) or tricyclic antidepressants are thought to have a beneficial influence on esophageal sensitivity (115, 116). A systematic review by Weijenborg *et al.* demonstrated that anti-depressive drugs can modulate esophageal sensation and can improve functional chest pain. However, review of the literature shows only limited evidence

that a subgroup of patients with rGERD would benefit from therapy with antidepressants (117).

In conclusion, rGERD is a complex disorder and its broad range of clinical manifestations does not facilitate the decision for optimal treatment of this patient population. As many factors are involved in heartburn perception, additional research is needed to investigate novel therapeutic approaches.

CHAPTER 2

RESEARCH OBJECTIVES

2 Research Objectives

2.1 Mechanisms contributing to symptom generation and symptom perception in patients with refractory GERD

The pathophysiology of refractory GERD (rGERD) symptoms remains incompletely understood. Although acid suppressive therapy with PPIs is the first option for treatment of heartburn, in 10 to 40% of the patients with typical GERD symptoms, PPI treatment is less effective than expected (54-56). In this PhD project, we focus on the involvement of esophageal hypersensitivity in rGERD patients on a double dose of acid suppressive therapy (proton pump inhibitors) and on the effect of stress on esophageal pain perception in health.

2.1.1 Inadequate acid suppression in refractory GERD patients on PPI therapy

Insufficient acid suppression and ongoing acid or weakly acid reflux is a potential cause of rGERD symptoms (55). Therefore, the very first step in unraveling the underlying mechanisms of rGERD symptoms was to study the characteristics of different reflux parameters assessed by 24 hour multichannel intraluminal impedance-pH monitoring in patients with rGERD on PPI therapy and to compare them with healthy controls. This part of the project was addressed in **Chapter 4** of the thesis.

2.1.2 Alteration in esophageal sensitivity in refractory GERD

Alterations in esophageal sensitivity are potentially involved in rGERD symptom generation (16, 60). We will investigate the presence of changes in esophageal sensitivity by using a multimodal esophageal stimulation paradigm where we will compare sensitivity to thermal, mechanical, electrical and chemical stimuli between rGERD patients on PPI therapy and healthy controls **(Chapter 4)**.

Another factor involved in changes in heartburn perception can be the up-regulation of acid sensing receptors mainly located on the nerve endings present between cells of the lower layers of the epithelium. In this project, we will investigate the expression and distribution of acid sensitive ion channels (ASIC) as well as the protease activated receptor 2 (PAR-2), the transient receptor potential vanilloid type-1 (TRPV1) and the delta subunit of the epithelial sodium channel (δENaC). It has already been shown that some of these receptors (mainly TRPV1 and PAR-2) are upregulated at mRNA and protein levels in GERD patients (76, 118). Nevertheless, more reliable methodologies to evaluate protein expression such as Western

blot are lacking to further confirm this suggested increase in expression levels. The aim of the current project was to investigate whether the expression of acid sensitive receptors was altered in rGERD patients on PPI therapy **(Chapter 4)**.

2.1.3 Changes in esophageal integrity underlying alterations in esophageal sensitivity

Dilated intercellular spaces (DIS) are reported to be a morphological marker in the pathogenesis of GERD, reflecting the alteration of esophageal epithelial integrity (119). Heartburn perception has been associated with presence of DIS in the esophageal epithelium, indicating that DIS facilitate the triggering of nerve endings by acid and other gastric compounds (79, 119).

Since impaired esophageal epithelial integrity can underlie changes in esophageal sensitivity, biopsy samples will be collected and esophageal integrity will be assessed using the Ussing chamber technique. Epithelial permeability and transepithelial electrical resistance (TEER) will be compared between patients with rGERD on PPI therapy and healthy controls **(Chapter 4)**.

2.1.4 Anti-nociceptive pathways involved in esophageal sensitivity in health

We hypothesized that esophageal sensitivity in rGERD could also be affected by a failure of the anti-nociceptive pathways involved in esophageal pain perception (120). To further unravel which neurotransmitters are involved in esophageal pain perception, three different pathways will be blocked in healthy controls to investigate if antagonizing these neurotransmitter systems can alter esophageal sensitivity and esophageal pain perception. In **Chapter 5** of this PhD project we focused on: i) the endogenous opioid system, ii) the serotoninergic system and iii) the dopaminergic system.

2.1.5 Alteration in esophageal pain perception due to stress

Central mechanisms, influenced by factors such as stress, anxiety, and personality traits, have all been implicated in the pathogenesis of esophageal hypersensitivity (65). Corticotropin-releasing hormone (CRH) is a key player in the response of the gastrointestinal tract to acute and chronic stress (121, 122). In **Chapter 6**, we therefore studied the influence of peripheral CRH on esophageal sensitivity and motility in health; we investigated whether we could alter esophageal sensitivity and motility by an acute peripheral administration of CRH.

CHAPTER 3

MATERIAL AND METHODS

3 Materials and methods

3.1 Study population

All study procedures were performed at the University Hospital of Leuven (Belgium) and were approved by the Ethics Committee of the University Hospital of Leuven. Written informed consent was obtained from all participants prior to initiation of the study and studies were in agreement with the latest version of the Declaration of Helsinki.

3.1.1 rGERD patient selection

Ambulatory patients between 18 and 70 years, presenting at the general gastroenterology outpatient clinic with typical symptoms of GERD (heartburn and/or regurgitation) refractory to at least 2 months high dose proton pump inhibitor therapy (b.i.d) were eligible for the studies. Patients were excluded if they had a history of former upper digestive or anti-reflux surgery, Barrett's esophagus, achalasia, esophageal outflow obstruction, Jackhammer esophagus, coeliac disease, inflammatory bowel disease. Pregnant or breastfeeding women were restrained from the studies.

3.1.2 Healthy subject selection

All healthy volunteers (HVs) were aged between 18-60 years and presented to the endoscopy unit after a fasting period of at least 6 hours. HVs were excluded if they had history of any gastrointestinal disease or any other comorbidities (neuromuscular, psychiatric, cardiovascular, pulmonary, endocrine, autoimmune, renal and liver disease), prior history of esophageal, ear-nose-throat or gastric surgery or endoscopic anti-reflux procedure. None of the subjects were taking any medication except for oral anti-contraceptives. Pregnant or breastfeeding women were restrained from the studies.

3.2 Ambulatory multichannel intraluminal impedance-pH monitoring

When a definite diagnosis of GERD is needed, 24 hour esophageal multichannel intraluminal impedance-pH monitoring (MII-pH) monitoring is currently considered the gold standard for the detection of reflux episodes (123). Esophageal MII-pH monitoring uses the measurement of electrical impedance between a pair of electrodes mounted on the impedance-pH catheter. Impedance is inversely proportional with the electrical conductivity of luminal contents (gas

or liquid). When a liquid bolus passes through the esophagus, a drop in impedance values can be detected, impedance values increase when gas is present.

The impedance-pH catheter (ComforTEC® Z/pH, Diversatec Healthcare, Highlands Ranch, CO, USA) consists of 6 impedance channels and 2 pH sensors and is placed transnasally with the proximal pH sensor positioned 5cm above the LES. In this way, the distal pH sensor is located in the stomach and impedance channels are positioned 3, 5, 7, 9, 15 and 17cm proximal to the LES. Impedance and pH measurements are performed at 50Hz and are recorded on an ambulatory device (ZepHr or Sleuth, Diversatec Healthcare, Highlands Ranch, CO, USA) (Figure 3.1).

Figure 3.1 Ambulatory devices used for MII-pH for reflux monitoring and schematic representation of the positioning of a multichannel intraluminal impedance-pH catheter in the distal esophagus.

Combined MII-pH monitoring, which became commercially available only a decade ago, has significantly increased the diagnostic yield and the sensitivity of reflux detection, as it enables detection of all types of reflux, independent of their acidity (acidic, weakly acidic, weakly alkaline). Additionally, it permits the detection of anterograde and retrograde bolus flow (liquid, gas or mixed) (123-125) (Figure 3.2).

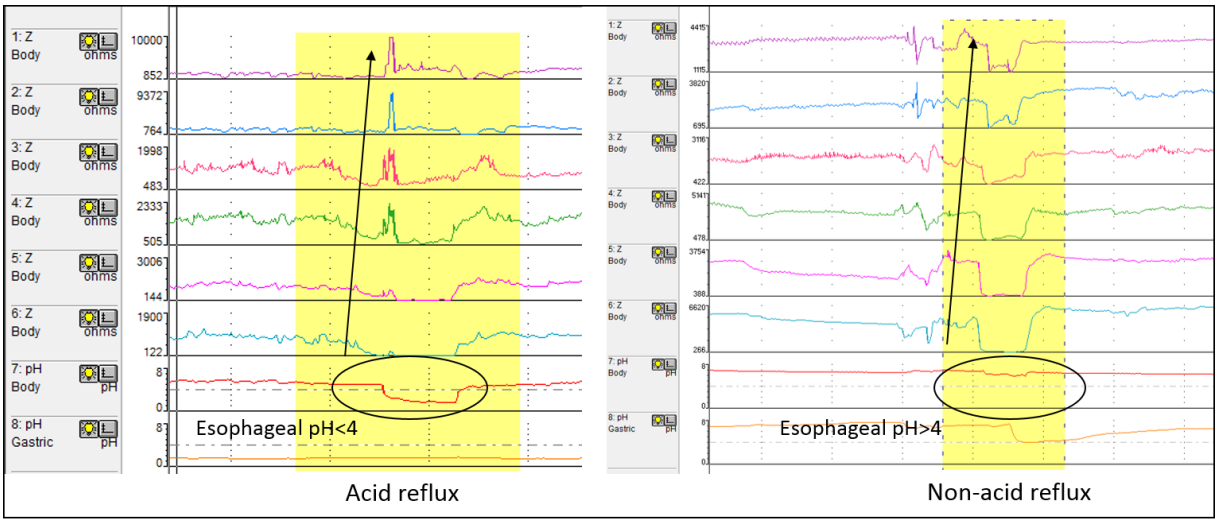

Figure 3.2 Example of an acid and non-acid reflux episode as detected with 24 hour MII-pH monitoring. The arrow indicates the retrograde direction of bolus flow, impedance drop is first detected in the most distal impedance channel and migrates upwards to the most proximal impedance channel.

According to international guidelines for analyzing MII-pH monitoring, liquid reflux was defined as a retrograde drop in impedance to less than 50% of baseline, starting in the most distal channel, propagating upwards to at least one more proximal impedance channel (19). Reflux was considered acidic when pH fell below 4 for at least 4 seconds. Weakly acidic reflux was defined as a reflux event with the basal pH remaining between 4 and 7 (19, 125). Meal times, posture changes (upright versus recumbent) and symptoms were recorded by the participants as event markers on the ambulatory device. The following reflux parameters were investigated: total 24 hour esophageal acid exposure time (AET, %time) defined as the total time period with esophageal pH less than 4 divided by the total monitoring time, total number of reflux events, number of acid and non-acid reflux events, percentage of mixed reflux events (combined gas and liquid reflux), total volume exposure time (%time) defined as the total time period of bolus exposure divided by the total monitoring time, and the proximal extent of reflux events defined as the proximal level to which the reflux event caused a drop of impedance. Reflux parameters were evaluated according to the normal range cut-off guidelines formulated by Zerbib *et al.* (20). Furthermore, the association between symptoms and the occurrence of reflux events was assessed by calculating two parameters: i) symptom association probability (SAP) and ii) symptom index (SI). A positive SAP was defined by a probability of 95% or more that symptoms were associated with reflux, and a positive SI was defined by 50% or more of symptoms associated with reflux (19, 20, 123).

3.3 Measurements of esophageal sensitivity: multimodal esophageal stimulation

To further unravel the pathways that are involved in heartburn perception, we used a multimodal esophageal pain evaluation approach. Esophageal sensitivity has been investigated in previous studies, revealing that thermal, mechanical, electrical and chemical stimuli can all be perceived in the esophagus. Since pain is a multidimensional experience, the optimal way to evaluate this sensation is to use a multimodal stimulation approach, as previously published (126, 127). We used a custom-made multimodal stimulation probe designed by the research group of Professor Asbjørn Drewes and Professor Hans Gregersen (Aalborg Hospital, Denmark) (Figure 3.3). This probe, equipped with a polyurethane balloon, allows studying esophageal sensitivity to four stimulation modalities: i) thermal stimulation ii) mechanical stimulation iii) electrical stimulation and iv) chemical stimulation.

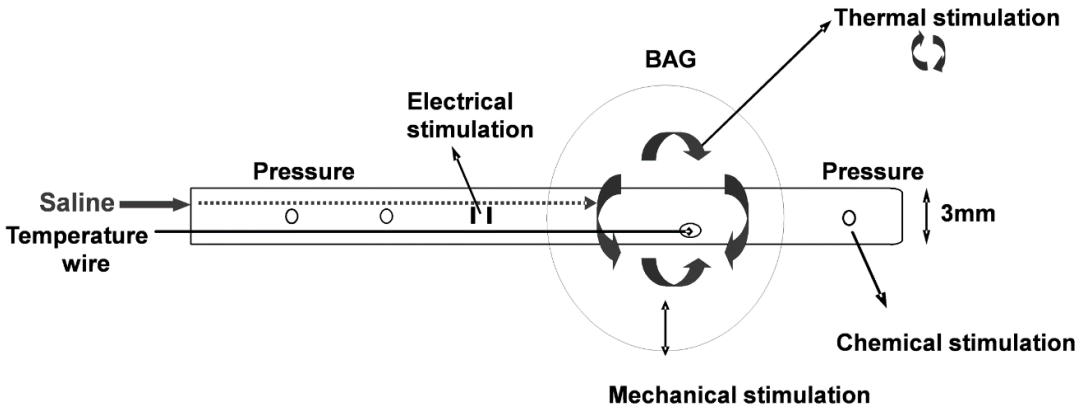

Figure 3.3 Schematic representation of the multimodal esophageal stimulation probe.

The multimodal stimulation probe was placed through the mouth with the top of the inflatable balloon positioned 10cm proximal to the LES (126). To locate the LES, the balloon was inserted into the stomach and filled with 20mL of saline with subsequent retraction of the probe to identify the LES. Subsequently, after deflating the balloon the probe was further retracted 10cm proximal to the LES (Figure 3.4). The subjects remained in a semi-recumbent position for the entire study period.

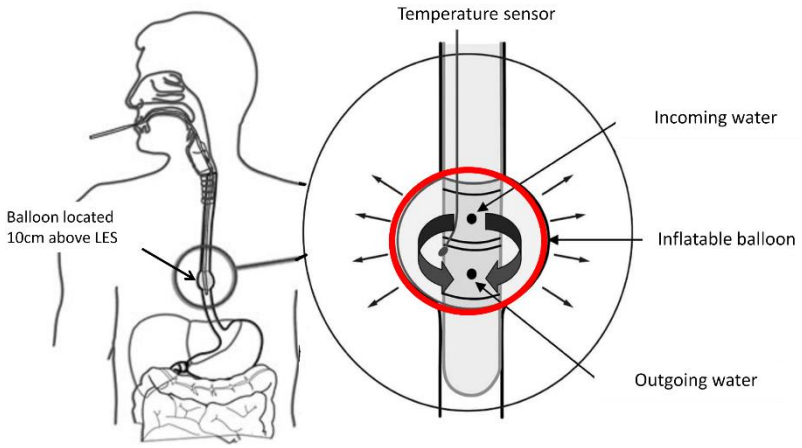

Figure 3.4 Schematic representation of the positioning of the multimodal stimulation probe via the mouth in the distal esophagus. The top of the inflatable balloon is located 10cm above the LES. Abbreviations: LES= lower esophageal sphincter. Adapted from Drewes A. *et al.* 2002 (126).

During the four different esophageal stimulation modalities, subjects were instructed to report perception of the stimuli using a pain scoring system (Figure 3.5), which has been shown to be reliable in discriminating esophageal sensations (128), to indicate the 1st perception threshold, pain perception threshold (PPT) and the pain tolerance threshold (PTT).

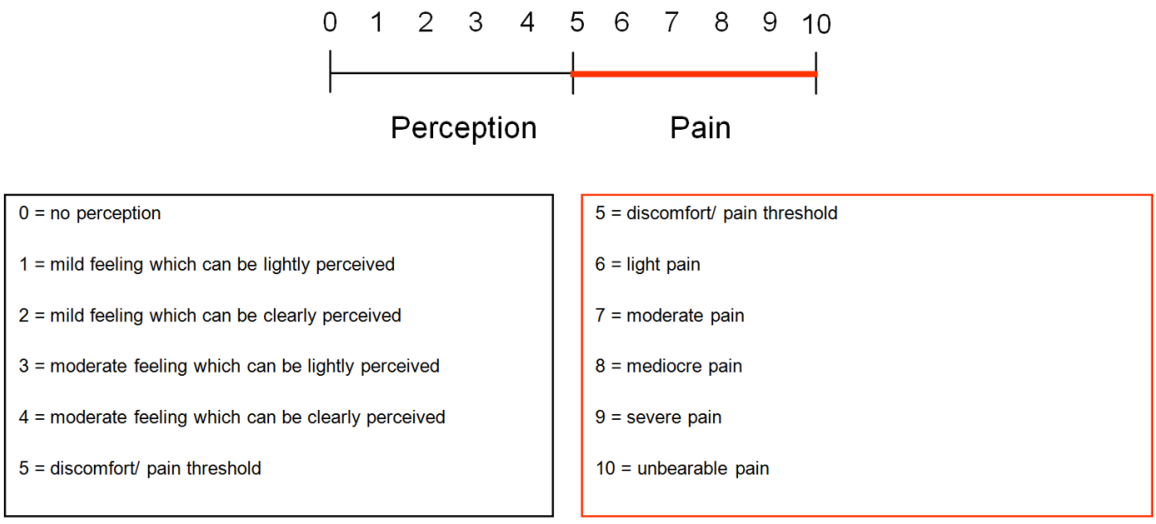

Figure 3.5 Pain scoring scale used during the multimodal esophageal stimulation protocol. First perception threshold, pain perception threshold (PPT) and pain tolerance threshold (PTT) were indicated by the study participants.

Thermal stimulation was performed by circulating a heated saline solution (NaCl 0.09%) through the balloon mounted on the probe. Infusion water was warmed by passage through a water bath with a maximal temperature of 62°C. The stimulation temperature was steadily increased by increasing the flow rate from the water bath to the balloon, flow rate was

controlled by a computer operated pump (Harvard PHD 2000). The volume in the balloon was kept constant at 5mL to avoid mechanical stimulation of the esophagus. A temperature sensor present in the balloon continuously monitored the stimulation temperature. The thermal stimulation was repeated three times with a 2 minute interval between the stimulation events. During all three stimulation sequences, subjects were asked to indicate their PPT and the stimulation was continued until the subject reached PTT.

Mechanical stimulation of the esophagus was implemented by distention of the balloon. The flow of a saline solution (NaCl 0.09%) into the balloon, inducing the distention, was regulated by a computer controlled pump (25mL/min, ramp distention). Mechanical stimulations were performed using a 0.09% saline solution of 37°C, to avoid thermal stimulation of the esophagus. The mechanical stimulation was repeated three times with a 2 minute interval. The first two stimulation events were terminated when the subject reached PPT. For the third event, the stimulation was terminated when the subject reached PTT.

Two electrodes mounted on the probe proximal to the inflatable balloon were used to administer short electrical pulses. Electrical block pulses with a duration of 1ms at 200Hz were given using a standard electrical stimulator (DS5 Isolated Bipolar Current Stimulator, Digitimer, Hertfordshire, United Kingdom) (126). The amplitude of the pulses was steadily increased, with steps of 0.5mA at an interval of 15 seconds. The maximum intensity was limited to 50mA, as previous studies have shown a potential for atrial capturing with higher intensities (126, 129). ECG monitoring was performed as a safety measure during the electrical stimulations. The electrical stimulation was repeated three times with a 1 minute interval between the stimulation events. During all three events, subjects were asked to indicate their 1st perception threshold and the stimulation was continued until the subject reached the PPT.

Chemical stimulation was performed by adapting the Bernstein test, used in clinical practice to diagnose GERD in the early sixties (130). Since the lumen for acid infusion is located beneath the balloon, the catheter was pulled upwards 3cm before the acidic solution (Hydrochloric acid, 0.1N) was infused in the distal esophagus. Chemical stimulation was controlled by a peristaltic infusion pump with a flow rate of 2mL/min. During the chemical stimulation, subjects were asked to indicate their 1st perception threshold, PPT and PTT. The stimulation

was performed only once and lasted for a maximum period of 30 minutes or was terminated when subjects reached PTT.

An assessment of general mood and psychological state was made using the Positive and Negative Affect Schedule (PANAS) and the State-Trait Anxiety Inventory (STAI-state) questionnaires (131, 132). The STAI-state is a validated and widely used questionnaire measuring levels of transitory anxiety (132). The scale consists of 20 items, which are answered on a 4-point scale. A total score was calculated according to the instructions of the questionnaire.

3.3.1 Reproducibility of the multimodal esophageal stimulation protocol

The multimodal esophageal stimulation model was validated by retrospective evaluation of control sessions of HV who participated in several multimodal esophageal stimulation protocols. Five independent control conditions were extracted from our own database which consists of over 40 HV and were evaluated retrospectively to assess the reproducibility of the multimodal esophageal stimulation protocol. Control conditions from 15 HV (8m/7f, mean age 33 years, range [23-51]) who participated in at least two different study days were analyzed. Comparative results are shown in Table 3.1.

Table 3.1 Five independent control conditions were compared to establish the reproducibility of the multimodal stimulation protocol. Control conditions of 15 HV who participated in at least two different studies were analyzed.

	Control 1 (n=10)	**Control 2** (n=11)	**Control 3** (n=8)	**Control 4** (n=6)	**Control 5** (n=10)	**p-value** uncorrected
Thermal stimulation (°C)						
PPT	46.94±1.24	46.77±1.19	47.95±1.35	46.84±1.53	48.24±1.24	0.72
PTT	49.31±1.17	49.14±1.12	49.79±1.28	49.05±1.46	50.15±1.17	0.92
Mechanical stimulation (mL)						
PPT	37.93±3.75	34.66±3.59	28.32±4.17	34.77±4.80	26.29±3.94	0.14
PTT	40.15±3.62	40.68±3.45	35.78±4.03	43.40±4.65	33.31±3.81	0.37
Electrical stimulation (mA)						
1st perception	9.38±0.86	6.87±0.77	5.65±0.94	9.43±1.20		0.02*
PPT	12.72±1.85	10.78±1.73	12.63±1.96	15.74±2.10		0.31
Chemical stimulation (mL)						
1st perception	21.07±4.52	19.00±3.99	19.64±4.37	27.90±5.09		0.45
PPT	29.82±5.81	30.40±5.60	36.22±5.63	36.47±9.83		0.87
PTT	39.17±5.78	38.16±5.27	34.04±6.43	44.15±12.53		0.90

Results are presented as mean± standard error. A p-value<0.05 was considered statistically significant. PPT= pain perception threshold, PTT=pain tolerance threshold. * Survives Bonferroni correction.

There were no significant differences between PPT and PTT for thermal and mechanical esophageal stimulation in any of the control conditions. Comparison of the electrical stimulation in the independent control conditions revealed an overall significant difference for the 1st perception threshold, which was higher on the first study (p=0.02). However, when the 4 control conditions were compared to each other using post-hoc analysis, this difference only reached borderline significance after applying Bonferroni correction for multiple testing (p=0.05). Values for PPT to electrical stimulation were similar in all control conditions. In the chemical stimulation no differences were found, the values for the 1st perception threshold, with similar PPT and PTT values in all the independent observations.

By comparing independent control conditions of healthy subjects, we demonstrated that the multimodal stimulation paradigm is reproducible, since there were no significant differences between 1st perception thresholds, PPT and PTT thresholds for thermal, mechanical and chemical esophageal stimulation in any of the control conditions. We only found a significant difference for the 1st perception threshold for electrical stimulation. However, values for PPT, which allow a discrimination between HV and patients with refractory GERD, were similar in

all conditions. In conclusion, these comparisons of different, independent control conditions show that our multimodal stimulation protocol generates reproducible perception scores in healthy subjects and therefore provides a reliable method to assess esophageal sensitivity changes after administration of drugs or other interventions that may alter esophageal sensitivity.

3.4 Esophageal biopsies

Biopsy specimens were taken with a biopsy forceps (Radial Jaw3, outside diameter 2.2mm; Boston Scientific, Natick, Massachussetts, USA) in the distal esophagus (5cm above the LES) and the proximal esophagus (5cm beneath the UES) by two experienced endoscopists (JT, TV) during upper endoscopy. Both from the proximal and distal area, two biopsies were snap frozen in liquid nitrogen for protein extraction and subsequent Western blot analysis. Two biopsies were fixed in formalin and embedded in paraffin for immunofluorescence. Three biopsy samples were kept in ice-cold oxygenated Krebs-Ringer bicarbonate buffer for Ussing chamber experiments.

3.4.1 Western blot analysis

Esophageal biopsy samples of approximately 10mg were snap frozen in liquid nitrogen and stored at -80°C. Total protein was extracted from of esophageal tissue using T-PER extraction buffer (Thermo Scientific, Waltham, USA). Protein concentration was determined by the Pierce bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL, USA) according to manufacturer's specifications. Equal amounts of protein were by separated a 4-12% sodium dodecyl sulphate/polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane.

Membranes were incubated overnight at 4°C with primary antibodies: rabbit monoclonal anti-PAR-2 (dilution 1:1000, Abcam, Cambridge, UK), rabbit polyclonal anti-TRPV1 (dilution 1:1000, Thermo Fisher Scientific, Rockford, IL, USA), mouse monoclonal anti-TRPV1 (dilution 1:1000, Sigma-Aldrich, Saint Louis, MO, USA), rabbit polyclonal anti-ASIC3 (dilution 1:500, Abcam, Cambridge, UK) or during 1 hour at room temperature with mouse anti-vinculin (1:10000; Sigma-Aldrich, Saint Louis, MO, USA), which served as the loading control. Secondary antibodies used were peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (both 1:5000, Thermo Fisher Scientific, Rockford, IL, USA); antibodies were incubated for 1 hour at

room temperature. Bands were quantified in a non-blinded manner by densitometry using ImageJ software (National Institutes of Health; https://imagej.nih.gov/ij/). Relative expression compared to the control group was calculated.

3.4.2 Immunofluorescence

Sections of 4µm were deparraffinized following general procedures, blocked with Protein Blocking Solution (Dako, Glostrup, Denmark) and incubated overnight at 4°C in rabbit anti-PAR-2 (1:100, Abcam, Cambridge, UK), rabbit anti-ASIC3 (1:100, Abcam, Cambridge, UK), rabbit polyclonal anti-TRPV1 (1:100, Thermo Fisher Scientific, Rockford, IL, USA) and rabbit polyclonal anti-δENaC (1:100, Thermo Fisher Scientific, Rockford, IL, USA). Secondary antibody used was donkey anti-rabbit IgG, Alexa Fluor 594 (1:1000, Thermo Fisher Scientific, Rockford, IL, USA). Tissues slides were incubated for 10 minutes with DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) (Thermo Fisher Scientific, Rockford, IL, USA). Representative confocal images were obtained with a LSM880 Laser Scanning Microscope at 63x magnification (Zeiss, Oberkochen, Germany).

3.4.3 Ussing chamber experiments

Esophageal biopsy samples were mounted in modified 3mL Ussing chambers (Mussler Scientific Instruments, Aachen, Germany) with an exposed area of 0.017 cm^2, as described previously (133). This methodology was used to measure epithelial integrity during the whole experiment, since it has been shown that epithelial biopsy samples are viable for 160 minutes in Ussing chambers (133).

Mucosal and serosal compartments were filled with 10mM glucose in Krebs–Ringer bicarbonate buffer. Solutions were kept at 37°C and continuously carbogenated with O_2/CO_2 (95/5%). Experiments were performed in open-circuit conditions. Transepithelial electrical resistance (TEER) was calculated according to Ohm's law (R=U/I) from the voltage deflections induced by bipolar constant-current pulses of 16µA every 6 seconds with duration of 200 milliseconds. TEER values were registered for each tissue at 30 minutes intervals over 2 hours. The average TEER values of all time points was taken and results are presented as $\Omega.cm^2$.

The paracellular probe fluorescein (molecular mass of 400Da, 20mg/200µl; Sigma-Aldrich, St Louis, Missouri, USA) was added to the mucosal compartment to quantify esophageal

permeability. Serosal samples were collected every 30 minutes over 2 hours, to measure passage of fluorescein, which was performed by measuring its fluorescence level using a fluorescence reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany). Since a paracellular probe needs time to accumulate on the serosal side, time points 0 and 30 minutes were left out of the analysis. The average of time points 60, 90 and 120 minutes of the 3 biopsy samples was taken, and passage of fluorescein is presented as pmol.

3.5 Standard high resolution impedance manometry

We used a solid-state high resolution impedance manometry (HRiM) catheter consisting of 36 pressure channels spaced at 1cm interval and 16 impedance channels (Unisensor AG, Attikon, Switzerland). The probe was placed transnasally and positioned along the esophagus with the tip of the catheter in the proximal stomach. Pressure and impedance data were acquired at 20Hz (Solar GI, Laborie, Mississauga, ON, Canada) (Figure 3.6).

Figure 3.6 Solid-state HRiM catheter consisting of 36 pressure channels spaced at 1cm interval and 16 impedance channels (Unisensor AG, Attikon, Switzerland). Pressure and impedance data were acquired at 20Hz (Solar GI, Laborie, Mississauga, ON, Canada). Abbreviation: HRiM=high resolution impedance manometry.

Recent advances in esophageal motility assessment include the shift from conventional manometry to high resolution manometry, and to visualization of esophageal pressures as topographic color plots instead of line tracings, which are less easy to interpret (Figure 3.7). The addition of impedance metrics to the manometry data allows to record patterns of bolus transport in relation to esophageal motor function without the need for simultaneous radiology (134). For this reason, we assessed esophageal motility using HRiM.

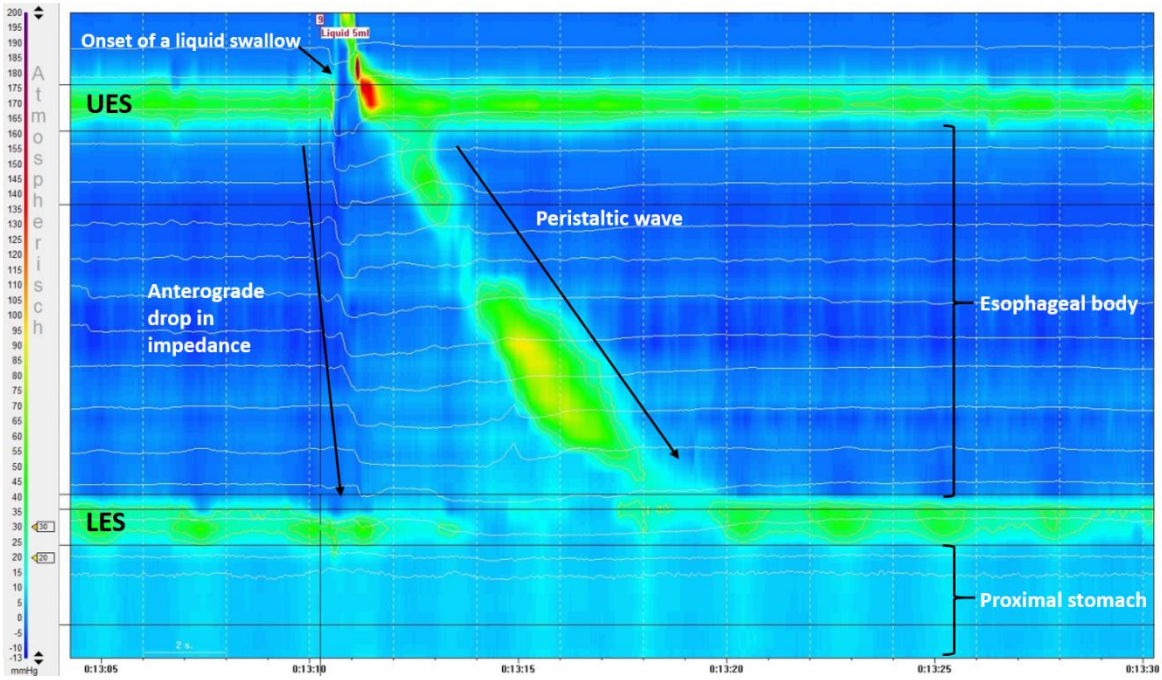

Figure 3.7 Example of a normal liquid swallow on a HRiM image. The pressure topographic picture represents intraluminal pressures encoded in colors corresponding to the scale on the left; low pressures are indicated by dark and light blue colors, higher pressures are indicated by yellow, red and purple colors. White lines indicate impedance metrics. Abbreviations: HRiM=High resolution impedance manometry, UES=Upper esophageal sphincter, LES=Lower esophageal sphincter.

Esophageal contractile function was evaluated by 10 liquid swallows of 5mL. During each of these 5mL swallows a number of different parameters were assessed including the distal contractile integral (DCI), measuring contractile vigor and the intrabolus pressure (IBP). Furthermore, the integrated relaxation pressure of the LES, mean of the 4 seconds of maximal deglutitive relaxation in the 10 second-window beginning at upper esophageal sphincter (UES) relaxation (IRP4) was calculated. IRP4 was used as a marker of resistance at the level of the esophagogastric junction (EGJ)/LES. Contraction patterns during these swallows were evaluated according to Chicago Classification v3.0, an international consensus to define esophageal motility disorders (135).

In routine clinical practice, esophageal peristaltic function is only assessed by 10 liquid swallows of 5mL. However, symptoms or complaints in patients are often not displayed when using these liquid test swallows only, therefore a manometry protocol with three different bolus consistencies is often applied in our center. In this 3-type bolus protocol 5mL liquid (water), 5mL semi-solid (apple sauce) and 2cm^2 solid (white bread) swallows are used to assess esophageal peristaltic function. All bolus stock contained 1% NaCl to enhance conductivity. Ten swallows of each consistency were executed. For each type of bolus consistency, data

gathered from multiple swallows were averaged for each subject. These mean values were used for further analysis (Solar GIHRM; Laborie, Mississauga, ON, Canada). Although the evaluation scheme of the Chicago Classification is based on the analysis of ten 5mL liquid swallows performed in supine position, we used the Chicago Classification for the analysis of liquid, semi-solid and solid swallows in a semi-recumbent position, as also done in our clinical routine.

Combined esophageal manometry and impedance recordings allow to describe the complex interplay between bolus transport and pressure generation. Therefore, pressure flow analysis was performed using esophageal automated impedance manometry software (AIMPlot_OES_V4.2, copyright T. Omari and N. Rommel, 2014), a purpose-designed analysis program written in MATLAB (version 7.9.0.529 R2009b, The Mathworks, Natick, MA, USA) (136).

Five space-time landmarks were defined on a standard pressure iso-contour plot of the esophageal swallow: i) time of onset of swallow, ii) time of proximal peak pressure, iii) proximal margin of the esophageal pressure wave sequence, iv) position of the transition zone, v) distal margin of the esophageal pressure wave sequence (Figure 3.8).

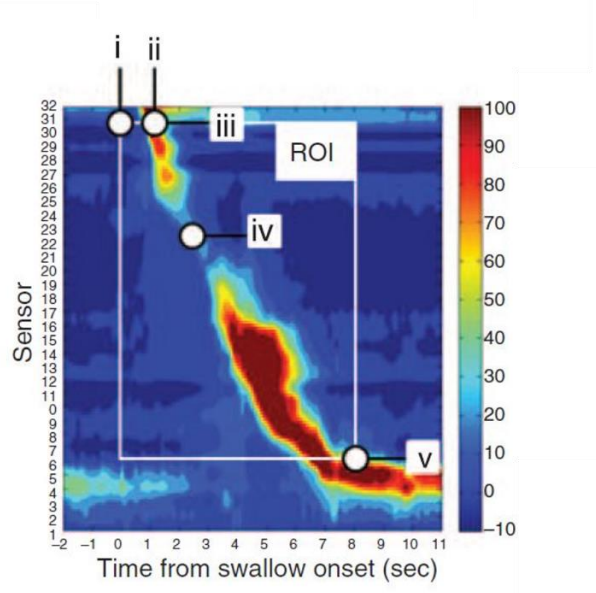

Figure 3.8 Esophageal pressure topography plot showing pressures associated with a 5mL liquid bolus swallow. Five space–time landmarks define the region of interest (ROI) for pressure flow analysis calculations. Adapted from Omari *et al.* (137)

The following parameters were evaluated: i) the ratio of nadir impedance to impedance at the time of peak pressure (impedance ratio, IR), which is used as a marker of bolus clearance, ii) the intrabolus pressure slope (IBP slope), the rate of change in IBP recorded during the phase of transition from a full lumen to an occluded lumen. IBP slope is a marker of the pressurization needed to propel a bolus forward and iii) pressure flow index (PFI) which reflects the relationship between intrabolus pressure and bolus flow timing in the esophagus. The PFI is calculated using the formula (IBPxIBP slope)/(time from nadir impedance to peak pressure) and serves as global measure of pressure flow or EGJ resistance to bolus flow (137, 138) (Table 3.2 and Figure 3.9).

Table 3.2 Description of all parameters used in pressure flow analysis.

PFA parameter	Unit	Interpretation
▪ **Nadir impedance (NI)**	Ohms	Bolus presence
▪ **Peak pressure (PP)**	mmHg	Pressure recorded at the moment of maximal contractile tension
▪ **Impedance at peak pressure (IPP)**	Ohms	Bolus presence at the time of maximal esophageal contractile tension
▪ **Impedance ratio (IR):** the ratio of nadir impedance to impedance at time of peak pressure		Marker of bolus clearance and incomplete bolus transit
▪ **Intrabolus pressure**	mmHg	IBP recorded during luminal emptying
▪ **Intrabolus pressure slope (IBP slope)**		Marker for pressurization needed to propel a bolus forward
▪ **Pressure flow index (PFI):** calculated according to the formula: IBPxIBP slope)/(time from nadir impedance to peak pressure)		Measure of pressure flow or EGJ resistance to bolus flow. The PFI describes the relationship between peristaltic strength and flow resistance in the distal esophagus

Adapted from Omari *et al.* 2014 (134).

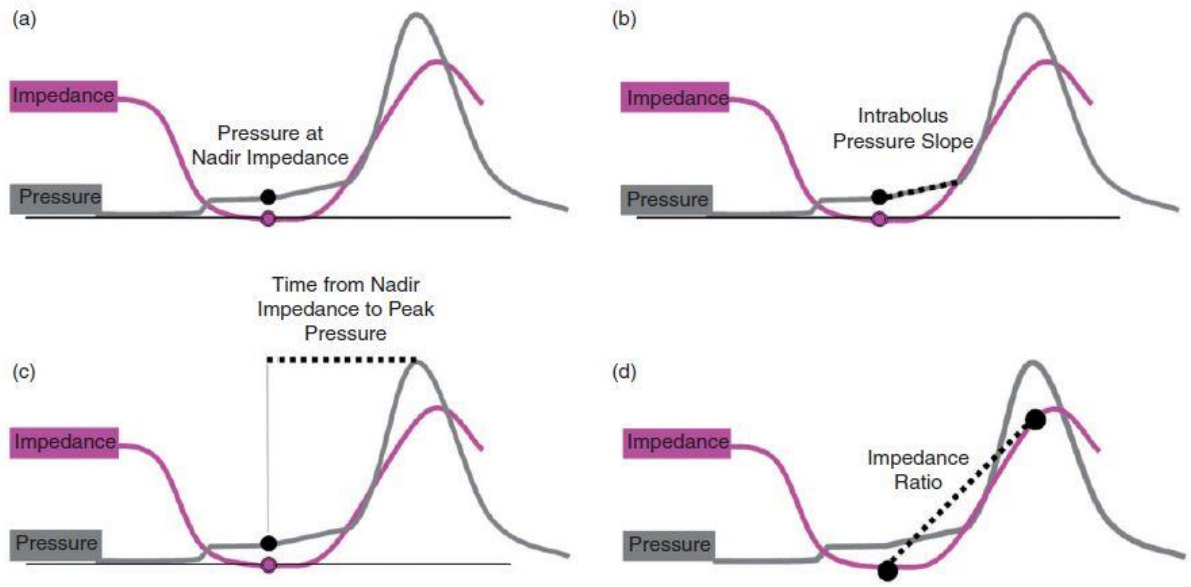

Figure 3.9 Pressure flow analysis using automated impedance manometric (AIMplot) software. **A)** Pressure at nadir impedance defines intrabolus distention pressures during bolus transport. **B)** Intrabolus pressure slope (IBP slope) the rate of change in IBP recorded during the phase of transition from a full lumen to an occluded lumen, IBP slope can serve as a marker for the degree of pressurization need to propel the bolus forward. **C)** Pressure flow index (PFI) is calculated by (IBPxIBP slope)/(time from nadir impedance to peak pressure). **D)** The ratio of nadir impedance to impedance at the time of peak pressure, impedance ratio (IR) is a marker of bolus clearance. Reproduced from Omari *et al.*, Copyright © 2014, © SAGE Publications (134).

CHAPTER 4

ALTERATIONS IN ESOPHAGEAL SENSITIVITY AND EPITHELIAL INTEGRITY IN REFRACTORY GERD

4 Alterations in esophageal sensitivity and epithelial integrity in refractory GERD

4.1 Introduction

GERD is a very prevalent, chronic disorder characterized by the frequent presence of typical symptoms (heartburn and/or regurgitation), however GERD can also manifest itself as atypical symptoms such as chronic cough, hoarseness or globus. Within the complex spectrum of GERD, two main phenotypes can be distinguished: patients with erosive esophagitis (EE), where erosions or ulcers are present in the distal esophagus on the one hand, and patients with non-erosive reflux disease (NERD) on the other hand. The latter subgroup represents the majority of reflux patients: up to 70% of the population with GERD symptoms does not show any macroscopic esophageal lesions during upper endoscopy (65, 139, 140).

A pivotal role for the acid component of the refluxate in the pathophysiology of GERD has already been demonstrated by experimental and esophageal pH-monitoring studies (141). Furthermore, the important influence of acidity of the refluxate is confirmed by the remarkable efficacy of acid-suppressive therapy in esophageal mucosal healing and the relief of symptoms in a large proportion of patients (2, 142). At present, proton pump inhibitors (PPIs) are still considered the most effective medical therapy for GERD (16, 143). Unfortunately, despite the fact that PPI therapy is very effective in acid suppression and in healing esophageal lesions, up to 40% of patients remains symptomatic even on double doses of PPIs (60, 144). In 2008, Bredenoord *et al.* suggested to use the term refractory GERD (rGERD) for the condition in which symptoms and/or mucosal lesions, caused by reflux of gastric contents, are not responding to high doses of PPI which implies a double dose of PPI for at least 12 weeks (57).

Patients with rGERD represent a very heterogeneous group and the reasons for these refractory symptoms can in part be attributed to lack of therapy compliance, insufficient acid suppression or ongoing weakly acidic reflux (55). However, the pathophysiological mechanisms of symptom generation in rGERD remain uncertain and the management of these refractory symptoms remains very challenging. Knowles and colleagues already suggested that differences in acid exposure time cannot be the only factor to explain symptoms of GERD

since the nature and severity of symptoms are very similar in patients with EE and patients with NERD (65).

Using a multimodal esophageal stimulation protocol in a small cohort of 10 rGERD patients on b.i.d. PPI therapy, our group was able to show that patients are hypersensitive to thermal, mechanical and chemical stimulation of the esophagus compared to healthy controls (145). These preliminary data further suggest that not only acid and weakly acidic reflux, but also visceral hypersensitivity can play a role in the generation and perception of reflux symptoms.

Visceral hypersensitivity may be caused by peripheral or central sensitization to stimuli in the GI tract. Peripheral sensitization can occur through increased expression of sensory receptors, or via an enhanced access of provocative stimuli to sensory structures on nerve endings. The presence of dilated intercellular spaces (DIS), which are reported to be an accurate morphological marker of impaired epithelial integrity in GERD (80), may attribute to peripheral sensitization since noxious stimuli are able to infiltrate the epithelial barrier. In addition, heartburn perception has been associated with presence of an impaired epithelial integrity shown by the presence of DIS in the esophageal epithelium (82, 83), indicating that acid and other (duodeno)-gastric compounds could cross the epithelium and reach nerve endings located between cells of the lower layers of the esophageal epithelium.

While the recurrent presence of acid and bile salts in the esophagus can lead to the development of DIS, Farré *et al.* demonstrated in rats that acute stress is also able to induce DIS and an increase in esophageal permeability (146). In addition, several clinical studies assessing the effect of experimental stressors on reflux perception indicate that there is an impact of psychosocial factors on symptom perception in GERD (87, 89, 147). Increased anxiety levels have been shown to decrease the thresholds for perception of visceral stimuli in several functional GI disorders such as functional dyspepsia (85). In a systematic review, Becher *et al.* described that the presence of GERD symptoms is associated with a decreased health-related quality of life (148) and also Kessing *et al.* demonstrated that increased anxiety levels are associated with more severe GERD symptoms (89).

An enhanced expression of sensory receptors may be an additional mechanism of peripheral sensitization which can lead to esophageal hypersensitivity. In recent studies, an upregulation of the expression of transient receptor potential vanilloid type-1 (TRPV1) and protease-

activated receptor 2 (PAR-2) was detected in esophageal mucosa of NERD patients, and these receptors are therefore suggested to play a role in symptom generation (70, 73). The expression and distribution of acid-sensitive ion channels (ASIC) in the esophageal epithelium remains to be further elucidated, but they are likely to be involved in chemosensitivity for acid (different ranges of pH) and mechanosensitivity (75).

Since NERD is the most frequent phenotype of GERD (65, 140), mechanisms of symptom perception in these patients with a macroscopically normal esophageal mucosa need further research. The aim of this study was to characterize patients with refractory GERD symptoms on b.i.d. PPI treatment and to investigate whether alterations in esophageal sensitivity and esophageal integrity are an underlying mechanism of rGERD symptoms. First of all, we assessed reflux parameters of rGERD patients on PPI therapy (b.i.d) and healthy controls to clarify if ongoing acid reflux or rather non-acid reflux and volume exposure are a possible contributing factor to symptom generation in these patients. Furthermore, multimodal esophageal stimulation was performed to confirm our earlier findings that esophageal sensitivity is altered in patients with rGERD compared to healthy controls. Esophageal epithelial integrity was assessed *in vitro* by Ussing chamber experiments and *in vivo* by performing impedance baseline measurements. In this way we can reveal possible changes in epithelial integrity and determine whether this could lead to esophageal hypersensitivity. Finally, esophageal biopsies were collected for investigations of the presence and distribution of acid sensitive receptors such as the transient receptor potential vanilloid type-1 (TRPV1), protease activated receptor 2 (PAR-2), acid sensitive ion channel 3 (ASIC3) and the delta subunit of the epithelial sodium channel (δENaC).

4.2 Materials and methods

4.2.1 Study population

Ambulatory patients between 18 and 70 years, presenting at the general gastroenterology outpatient clinic with typical symptoms of GERD (heartburn and/or regurgitation) refractory to PPI therapy (b.i.d) were eligible for the study. Patients were excluded if they had a history of former upper digestive or anti-reflux surgery, diabetes, irritable bowel syndrome, coeliac disease and inflammatory bowel disease. Pregnant or breastfeeding women were restrained from the study.

We also recruited healthy volunteers between 18-60 years for this study. Participants were excluded if they had any history of GI disease or any other comorbidities (neuromuscular, psychiatric, cardiovascular, pulmonary, endocrine, autoimmune, renal and liver disease), prior history of esophageal, ear-nose-throat or gastric surgery or endoscopic anti-reflux procedure. Pregnant or breastfeeding women were restrained from the study.

4.2.2 Study design

The outline of the study is depicted in Figure 4.1. At day one of the protocol, rGERD participants on a double dose of PPI were expected at the endoscopy unit of the university hospital after a fasting period of at least 6 hours. During routine upper endoscopy, we collected proximal (5cm beneath the UES) and distal (5cm above the LES) esophageal biopsies to study esophageal epithelial integrity. Three proximal and three distal biopsies were kept in ice-cold Hanks' Balanced Salt Solution (HBSS) buffer for Ussing chamber experiments, two biopsy specimens were snap frozen in liquid nitrogen for Western blot analysis, and two biopsies were stored in fixation buffer for immunofluorescence.

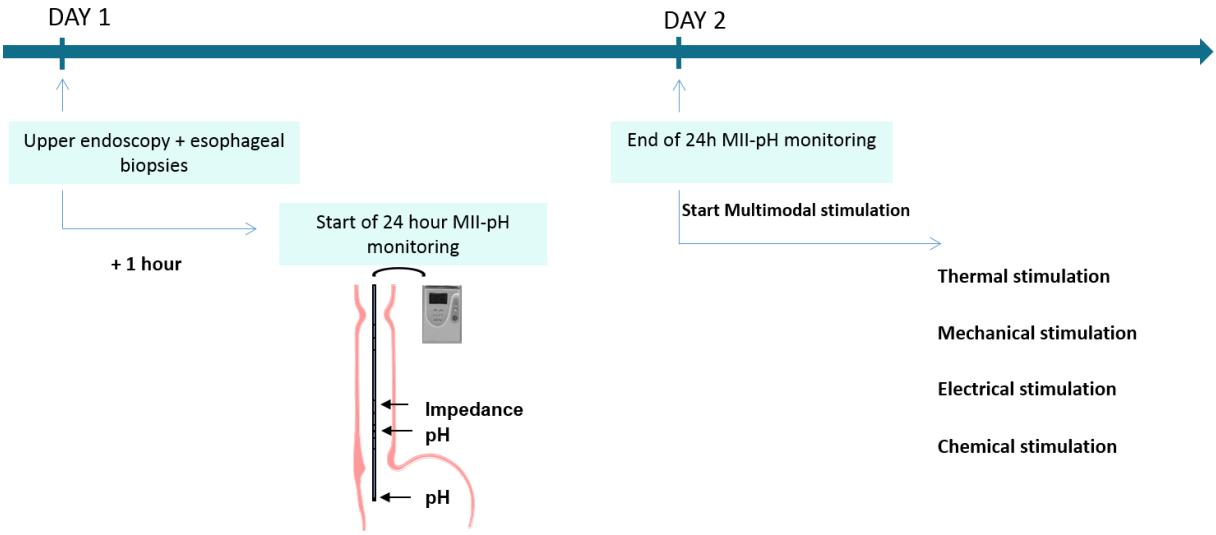

Figure 4.1 Outline of the study protocol. At day 1, study participants underwent an upper endoscopy which included the collection of proximal and distal esophageal biopsies. Hereafter, 24 hour MII-pH monitoring was initiated. At day 2, multimodal esophageal stimulation was performed after termination of the reflux monitoring. MII-pH=multichannel intraluminal impedance-pH.

After upper endoscopy, ambulatory 24 hour MII-pH monitoring was initiated to assess reflux characteristics of the study participants (described in Chapter 3, paragraph 3.2). The impedance-pH catheter was placed transnasally with the proximal pH-sensor positioned 5cm above the LES and the distal pH-sensor in the stomach. Reflux was considered acid when pH fell below 4 for at least 4 seconds. Weakly acidic reflux was defined as a reflux event with the basal pH remaining between 4 and 7 (125), cut-off values proposed by Zerbib *et al.* were used to determine presence of abnormal reflux parameters (20). The following parameters were evaluated: acid exposure time (%time), total number of reflux events, total number of acid and non-acid reflux events, total volume exposure time (%time), the number of reflux events with a high proximal extent (15cm above the LES or higher) and the percentage of mixed reflux events (combined gas and liquid reflux) . Furthermore, symptom association probability (SAP) and symptom index (SI) were calculated for rGERD patients.

At day 2 when MII-pH monitoring was terminated, HV and patients were involved in the multimodal stimulation protocol (described in Chapter 3, paragraph 3.3). An assessment of general mood and psychological state was made before and after the stimulation test by means of the Positive and Negative Affect Schedule (PANAS) and the State-Trait Anxiety Inventory (STAI-state) questionnaires (131, 132). In addition, study participants were asked to indicate the area where the stimulus was felt on a schematic drawing of the chest (Figure 4.2). The distance from the start of the sternum until the referred pain area where the stimulus

was perceived, was measured for each stimulation modality and compared between HV and rGERD patients.

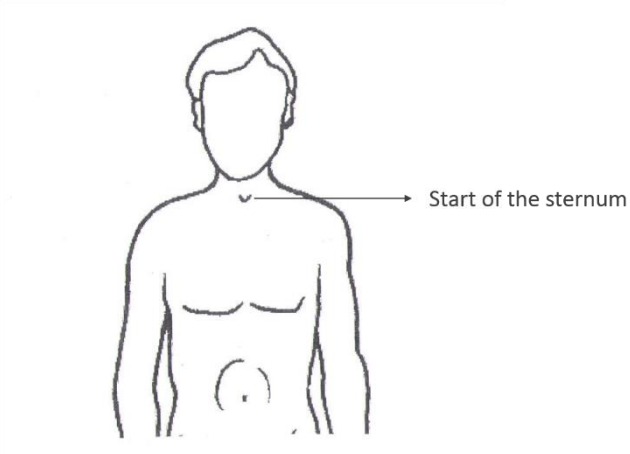

Figure 4.2 Indication of the referred pain area during the four esophageal stimulation modalities.

In vitro assessment of esophageal epithelial integrity was performed by Ussing chamber experiments. Biopsies collected during endoscopy at day 1 were mounted in modified 3mL Ussing chambers to evaluate transepithelial electrical resistance (TEER) and passage of fluorescein (Fl) as described in Chapter 3, paragraph 3.4.3. Mucosal and serosal compartments were filled with 10mM glucose in Krebs–Ringer bicarbonate buffer. TEER ($\Omega.cm^2$) was recorded every 30 minutes over 2 hours. After a stabilization period of 30 minutes, fluorescein was added to the mucosal compartment. Serosal samples were collected every 30 minutes over 2 hours, to measure passage of fluorescein (pmol).

In addition, esophageal integrity was also assessed *in vivo* with the multichannel intraluminal impedance technique. Impedance baseline was assessed using Bioview analysis software: reflux tracings were set at a time window of 30 minutes. Measurements were initiated one hour after the placement of the catheter and were performed at 3, 5 and 15cm above the LES in the upright position. In the beginning and the end of every 30 minute time window, two steady periods of 30 seconds were selected to assess impedance baseline, avoiding periods of swallowing, reflux events and pH-drops. Mean impedance baseline values were calculated for the three sites (3, 5 and 15cm).

As described in Chapter 3, paragraph 3.4, immunofluorescence was used to verify the presence of TRPV1, ASIC3, PAR-2 and δENaC receptors in esophageal epithelium.

Furthermore, Western blot analysis was performed to evaluate the presence of these receptors in the epithelial layers of the esophagus.

4.2.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.02 (GraphPad Software, Inc., La Jolla, CA USA). Differences between groups were tested by two-tailed unpaired Student's *t*-tests or Mann Whitney U tests depending on the distribution of the data which was evaluated using the Kolmogorov-Smirnov test. One-way ANOVA with post-hoc *t*-test was used to verify differences between subgroups of rGERD patients and HV. Two-way ANOVA with a post-hoc *t*-test per time point with Bonferroni correction for multiple testing was used to evaluate changes in emotional status over time in the two groups (PANAS and STAI state questionnaire data). Data are presented as median [25^{th}-75^{th} percentile], unless stated otherwise. Correlations were tested by calculation of the Pearson's or Spearman's correlation coefficient, depending on data distribution. Categorical values were compared using the Fisher's exact test.

4.2.4 Ethical approval

The protocol was approved by the ethics committee of the University Hospital of Leuven (approval number: S54004 and S52720). Written informed consent was obtained from all participants before inclusion in the study.

4.3 Results

4.3.1 Assessment of reflux parameters in HV and rGERD patients

Twenty-four hour MII-pH monitoring was performed in 26 rGERD patients with typical reflux symptoms, all on PPI therapy (b.i.d) (9m/17f, mean age 42 years, [range 19-66]) and 23 HV (9m/14f, mean age 24 years, [range 19-48]) (Table 4.1).

Table 4.1 Demographic data of rGERD patients on PPI and healthy controls.

Demographics	**rGERD on PPI n=26**	**HV n=23**	**p-value**
Mean age (range), years	42 (19-66)	24 (19-48)	**<0.0001**
Female gender n (%)	17 (65%)	14 (61%)	0.77
Body Mass Index (25-75th percentile)	24.91 (21.41-26.12)	23.72 (20.72-25.09)	0.17
Only heartburn, no regurgitation n (%)	11 (42%)		
Only regurgitation, no heartburn n (%)	7 (27%)		
Heartburn and regurgitation n (%)	8 (31%)		

Based on the cut-off values published by Zerbib *et al.* (20), total acid exposure time was within the normal range in all healthy controls, however the total number of reflux events was above the upper limit of normal (total number of reflux events >53) in 8 asymptomatic HV with normal endoscopy.

When reflux parameters were compared between the two groups, we found that although total acid exposure (%time) was slightly higher in the patient group, there was no statistical difference between rGERD patients on PPI and HV (2.09% [0.16-6.32] vs. 0.48% [0.19-1.12], p=0.13), suggesting that acid is well suppressed in our patient cohort (Table 4.2).

The total number of reflux events tended to be higher in rGERD patients on PPI compared to HV (54 [38-85] vs. 31 [18-60], p=0.05). The number of acid reflux events was similar in rGERD patients on PPI compared to HV (21 [6-42] vs. 23 [12-46], p=0.84). On the contrary, the number of non-acid reflux events was significantly higher in rGERD patients on PPI compared to HV (28 [13-44] vs. 15 [5-19], p=0.002). Total volume exposure (%time) was also higher in patients compared to HV (1.42% [0.60-1.95] vs. 0.50% [0.25-0.90], p=0.001). When comparing the number of proximal reflux events between patients and HV, we found a higher amount of proximal reflux events in patients compared to healthy controls (15 [5-24] vs. 4 [0-16], p=0.01).

No differences were observed in the percentage of mixed reflux episodes between rGERD on PPI and HV (42.86% [5.38-70.50] vs. 25.45 % [12.90-37.29], p=0.31) (Table 4.2). However, in rGERD the percentage of mixed and liquid reflux was similar (42.8% mixed reflux vs. 57.1% liquid reflux, p=0.12), while in HV the number of mixed reflux events was significantly lower compared to the number of liquid reflux events (25.45% mixed reflux vs. 74.55% liquid reflux, p=0.0019).

Table 4.2: Results of 24 hour MII-pH monitoring in rGERD on PPI and healthy controls.

Reflux parameters	**rGERD on PPI**	**Healthy controls**	**Uncorrected p-value**
AET (% time)	2.09 [0.16-6.32]	0.48 [0.19-1.12]	0.13
Total number of reflux events (n)	54 [38-85]	31 [18-60]	0.05
Number of acid reflux events (n)	21 [6-42]	23 [12-46]	0.84
Number of non-acid reflux events (n)	28 [13-44]	15 [5-19]	**0.002**
Total volume exposure (% time)	1.42 [0.60-1.95]	0.50 [0.25-0.90]	**0.001**
Number of reflux with proximal extent (n)	15 [5-24]	4 [1-16]	**0.01**
Mixed reflux events (%)	42.86 [5.38-70.50]	25.45 [12.90-37.29]	0.31

Data are presented as median [25^{th}-75^{th} percentile]. Abbreviations: AET= acid exposure time.

Based on the results of the upper endoscopy and 24 hour MII-pH monitoring, we were able to further subdivide our cohort of rGERD patients into patients with EE, NERD, reflux hypersensitivity and FH. After endoscopy, 3 out of 26 patients were diagnosed with erosive esophagitis (grade A or B) despite double dose PPI therapy. Two patients with erosive esophagitis had a hiatal hernia. In the group of patients with a normal endoscopy, there were six patients with a hiatal hernia. Twenty-three patients with typical GERD symptoms had a normal endoscopy. Based on reflux parameters and symptom association analysis we identified 14 patients with NERD, 4 patients with reflux hypersensitivity and 5 patients with FH (Figure 4.3).

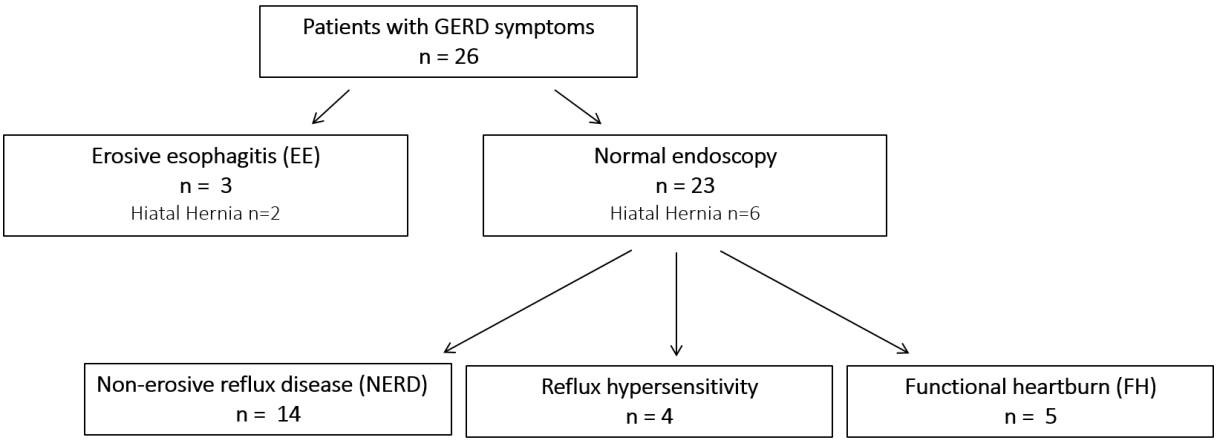

Figure 4.3 Based on upper endoscopy findings and the outcome of 24 hour MII-pH monitoring our cohort of rGERD patients was divided into different subgroups.

Symptom association analysis was performed by calculation of the SAP and SI. We found that 12 out 26 (46.15%) rGERD patients had a positive SAP and/or SI for acid reflux events. For non-acid reflux events, 15 out of 22 (57.69%) rGERD patients had a positive SAP and/or SI. Furthermore, we investigated whether the percentage of mixed reflux and liquid reflux events in patients with a positive symptom association was similar. In patients with a positive SAP and/or SI, the percentage of mixed reflux events was 39.9% compared to 60% of liquid reflux events (p=0.09).

Since our patient group was significantly older than the healthy control group we investigated the effect of age on reflux parameters. Therefore, data of rGERD patients and HV were pooled. When age was correlated to the reflux parameters of interest we found no significant associations (Table 4.3).

Table 4.3: Correlation of age and reflux parameters assessed by 24 hour MII-pH monitoring

Correlation	**Spearman correlation coefficient r**	**Uncorrected p-value**
Age vs. AET (% time)	0.08	0.58
Age vs. Total number of reflux events (n)	0.03	0.85
Age vs. Number of acid reflux events (n)	-0.07	0.61
Age vs. Number of non-acid reflux events (n)	0.16	0.26
Age vs. Total volume exposure (% time)	0.20	0.16
Age vs. Number of reflux with proximal extent (n)	0.03	0.82

Abbreviations: AET= acid exposure time

4.3.2 Alteration in esophageal sensitivity in refractory GERD

Twenty-three rGERD patients on PPI (9m/14f, mean age 42 years, [range 19-66]) and 23 HV (10m/13f, mean age 23 years, [range 19-39]) participated in the multimodal esophageal stimulation protocol. As mentioned above, the population of rGERD patients on PPI was significantly older than the healthy control group ($p<0.0001$). However, no significant correlations were observed between age and the multimodal stimulation modalities except for the PTT during chemical stimulation (Table 4.4).

Table 4.4: Correlation of age and multimodal stimulation modalities

Correlation	**Spearman correlation coefficient r**	**Uncorrected p-value**
Age vs. Temperature stimulation PPT	-0.19	0.22
Age vs. Temperature stimulation PTT	-0.19	0.23
Age vs. Mechanical stimulation PPT	-0.05	0.77
Age vs. Mechanical stimulation PTT	-0.02	0.90
Age vs. Electrical stimulation 1st Perception	0.13	0.42
Age vs. Electrical stimulation PPT	-0.04	0.78
Age vs. Chemical stimulation 1st Perception	-0.0006	>0.9999
Age vs. Chemical stimulation PPT	-0.22	0.15
Age vs. Chemical stimulation PTT	-0.33	**0.04**

Abbreviations: PPT=pain perception threshold, PTT=pain tolerance threshold

A negative association was found between increasing age and the volume of acid infusion at PTT ($r= -0.33$, $p= 0.04$), but significance was lost after Bonferroni correction (Figure 4.4).

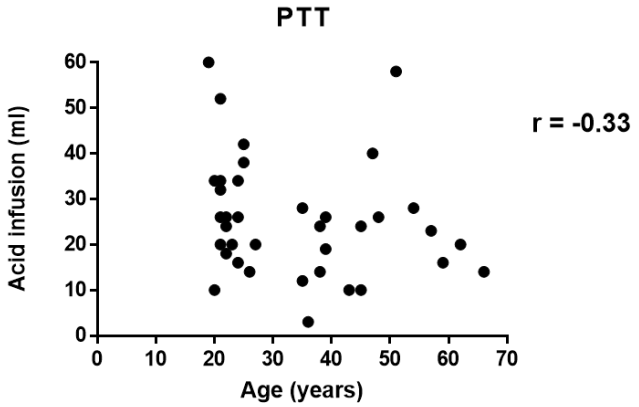

Figure 4.4 Negative correlation between the volume of acid infusion at PTT and age of HV and rGERD on PPI. Abbreviations: PTT=pain tolerance threshold.

As shown in Figure 4.5A and B, measurements for temperature stimulation were similar in rGERD on PPI and HV (PPT: 44.79°C [42.80-47.27] vs. 46.72°C [43.80-48.56] p=0.30, PTT: 46.87°C [45.40-50.92] vs. 49.02°C [46.00-51.31] p=0.39).

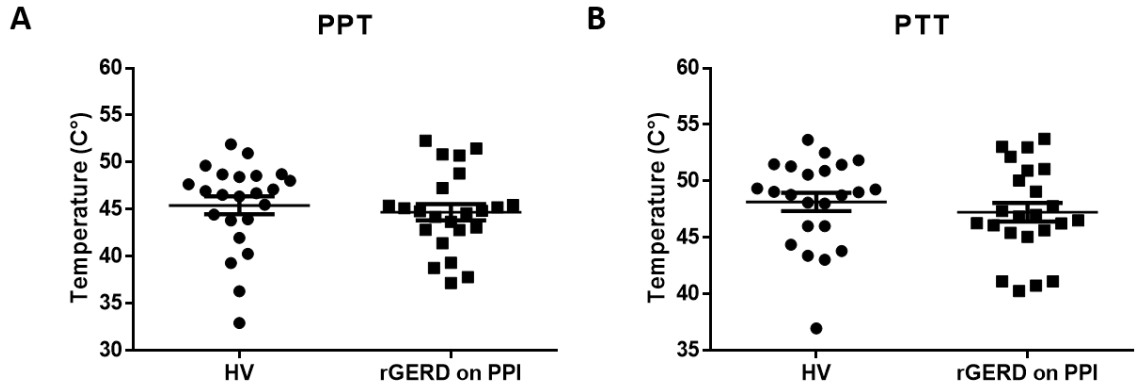

Figure 4.5 Results of **A)** PPT and **B)** PTT to esophageal temperature stimulation in HV and rGERD on PPI. No alterations in esophageal sensitivity to thermal stimulation were present when the two groups were compared. Abbreviations: PPT= pain perception threshold, PTT=pain tolerance threshold.

When we compared the pain perception threshold and the pain tolerance threshold for the mechanical stimulation, no differences in sensitivity thresholds were present between our cohort of rGERD on PPI and HV (PPT: 17.58mL [12.59-20.66] vs. 19.59 mL [12.78-24.50] p=0.13, PTT: 18.75mL [15.20-26.83] vs. 25.00mL [20.2-29.15] p=0.25) (Figure 4.6 A,B).

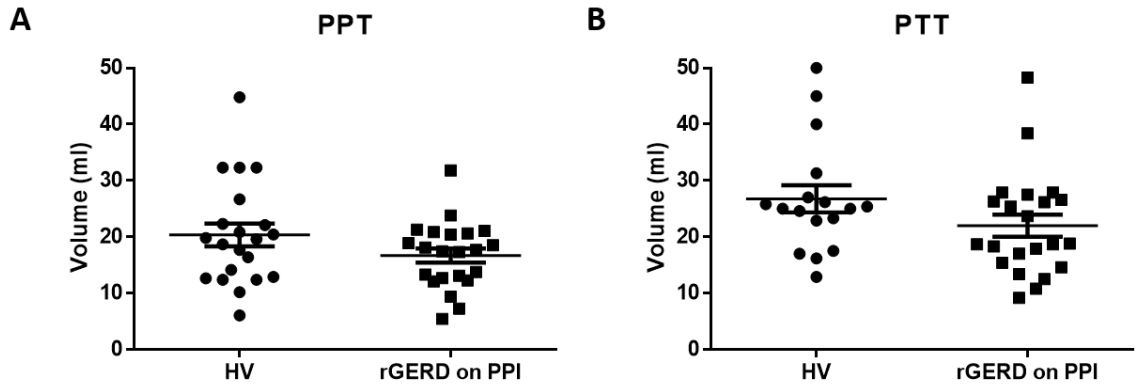

Figure 4.6 Results of **A)** PPT and **B)** PTT to esophageal mechanical stimulation in HV and rGERD on PPI. No alterations in esophageal sensitivity to mechanical stimulation could be demonstrated when the two groups were compared. Abbreviations: PPT= pain perception threshold, PTT=pain tolerance threshold.

The 1st perception threshold for electrical stimulation was similar in rGERD on PPI compared to HV (4.67mA [3.87-8.08] vs. 6.25mA [4.54-9.17], p=0.26) (Figure 4.7A). On the contrary, the pain perception threshold was slightly lower in rGERD on PPI compared to HV (9.75mA [7.34-15.34] vs. 13.00mA [10.00-18.21], p=0.04) (Figure 4.7B).

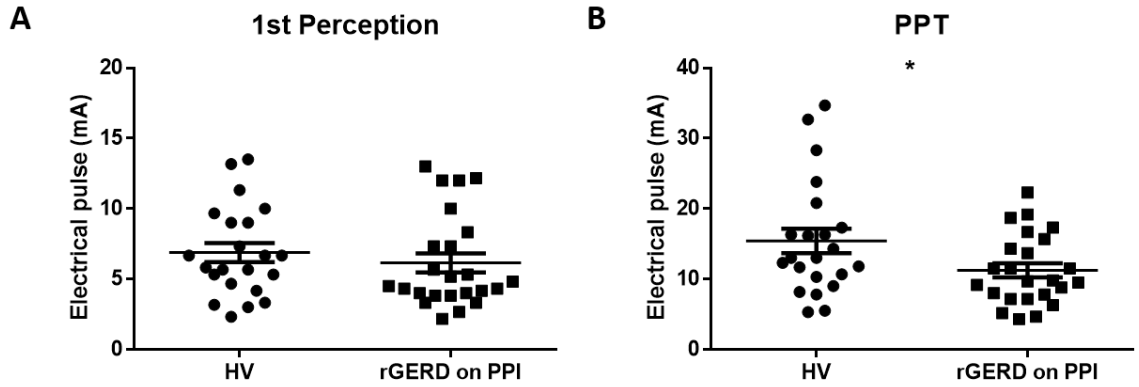

Figure 4.7 Results of esophageal electrical stimulation in HV and rGERD on PPI. **A)** No alterations in esophageal sensitivity to electrical stimulation were present for the 1st perception threshold. **B)** When the pain perception threshold was compared between the two groups, a significantly lower PPT was observed in rGERD patients. Abbreviations: PPT= pain perception threshold.

Finally, when acid sensitivity was assessed during chemical stimulation by infusion of 0.1N HCl, no differences in the 1st perception threshold were present between rGERD on PPI and HV (9.00mL [6.00-12.00] vs. 12.00mL [8.00-16.00], p=0.14) (Figure 4.8A). However, the threshold for pain perception was significantly lower in rGERD patients compared to HV (PPT: 14.00mL [8.00-24.00] vs. 22.00mL [18.00-30.00], p=0.01) (Figure 4.8B). Differences between rGERD and HV were even more pronounced for the pain tolerance threshold (PTT: 20mL [13.00-27.00] vs. 32.00mL [22.50-34.00], p=0.004) (Figure 4.8C).

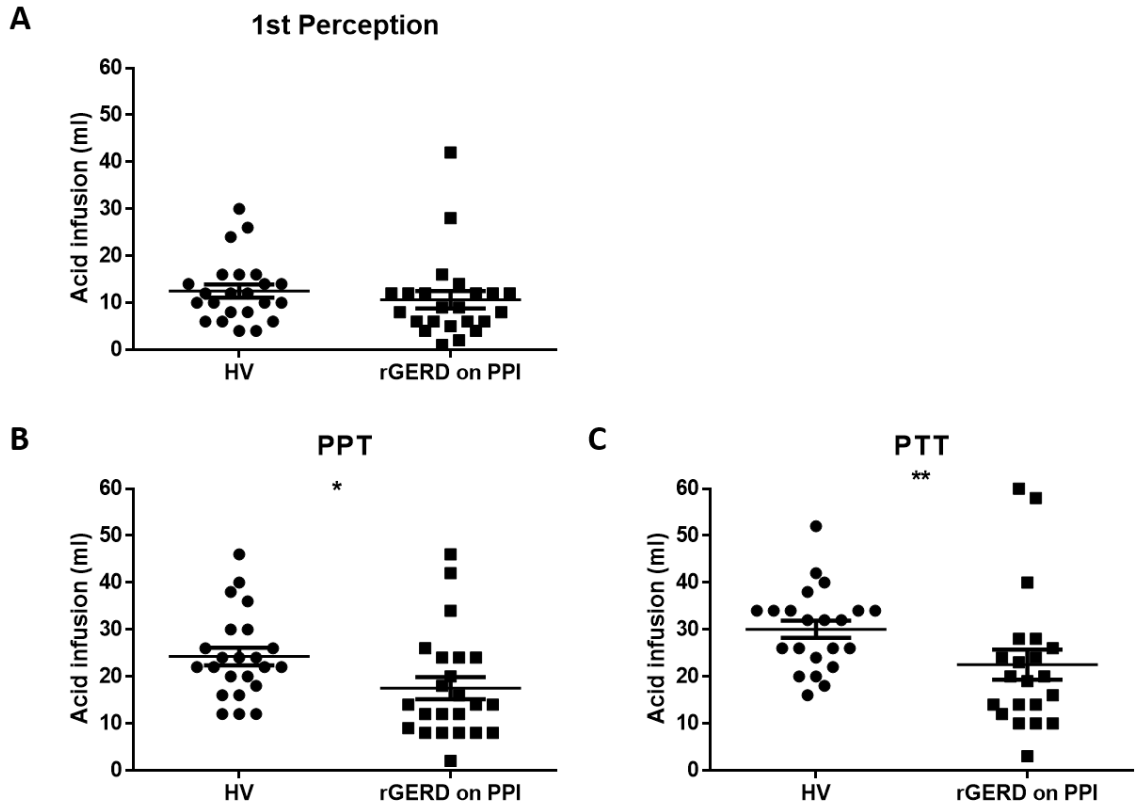

Figure 4.8 Results of esophageal chemical stimulation in HV and rGERD on PPI. **A)** Esophageal sensitivity to 1st perception threshold was similar in HV and rGERD on PPI. **B)** PPT and **C)** PTT were significantly lower in rGERD on PPI compared to HV. *p<0.05, **p<0.01. Abbreviations: PPT= pain perception threshold, PTT=pain tolerance threshold.

Based on the results of the symptom association analysis assessed by MII-pH monitoring, rGERD patients were subdivided into patients with a positive and negative symptom association probability (SAP+ and SAP-). Our patient cohort consisted of 11 SAP+ and 12 SAP- patients. Esophageal sensitivity to multimodal stimulation in these two subgroups was compared to the results of the multimodal stimulation in HV (Table 4.5). One-way ANOVA revealed a significant difference between HV, SAP+ and SAP- for the PPT during chemical stimulation and a borderline significant difference between PTT (Table 4.5).

Table 4.5 Results of esophageal multimodal stimulation in rGERD patients subdivided into SAP+ and SAP- patient groups.

	SAP +	SAP -	HV	p-value$_{uncorrected}$
Temperature (°C)				
PPT	45.39 [42.80-50.83]	44.38 [40.19-45.20]	46.72 [43.8-48.65]	0.16
PTT	47.79 [46.51-52.99]	45.86 [42.10-48.65]	49.02 [46.00-51.31]	0.07
Mechanical (mL)				
PPT	15.30 [11.43-19.19]	18.50[13.26-20.79]	19.59 [12.78-24.50]	0.23
PTT	22.10 [15.20-26.83]	18.70 [13.63-27.48]	25.00 [20.20-29.15]	0.47
Electrical (mA)				
1st perception	4.42 [3.83-7.67]	5.25 [4.04-9.33]	6.25 [4.54-9.17]	0.45
PPT	10.50 [8.21-14.17]	9.75 [7.17-16.42]	13.00 [10.00-18.21]	0.16
Chemical (mL)				
1st perception	12.00 [6.00-12.00]	7.00 [4.50-12.00]	12.00 [8.00-16.00]	0.20
PPT	16.00 [8.00-24.00]	13.00 [8.25-19.50]	22.00 [18.00-30.00]	**0.02**
PTT	21.00 [13.00-35.50]	20.00 [12.00-26.00]	32.00 [23.50-34.00]	0.06

Results are presented as median [25th-75th percentile]. HV: n=23, SAP+: n=11, SAP-: n=12. One-way ANOVA or Kruskal-Wallis test, post-hoc *t*-tests were performed with Bonferroni correction for multiple testing. SAP=symptom association analysis, PPT=pain perception threshold, PTT=pain tolerance threshold.

Post-hoc analysis revealed that during chemical stimulation, the PPT was similar in SAP- and SAP+ patients (p=0.41). When SAP- patients were compared to HV, we found a significantly lower PPT in SAP- patients (p=0.004) (Figure 4.9A). We observed a numerically lower PTT in SAP+ and SAP- patients compared to HV (p=0.06), however this difference did not reach significance (Figure 4.9B). SAP- patients had a significantly lower PTT than HV (p=0.005). No significant differences could be demonstrated between PTT for chemical stimulation in SAP+ and SAP- patients (p=0.59) (Figure 4.9B).

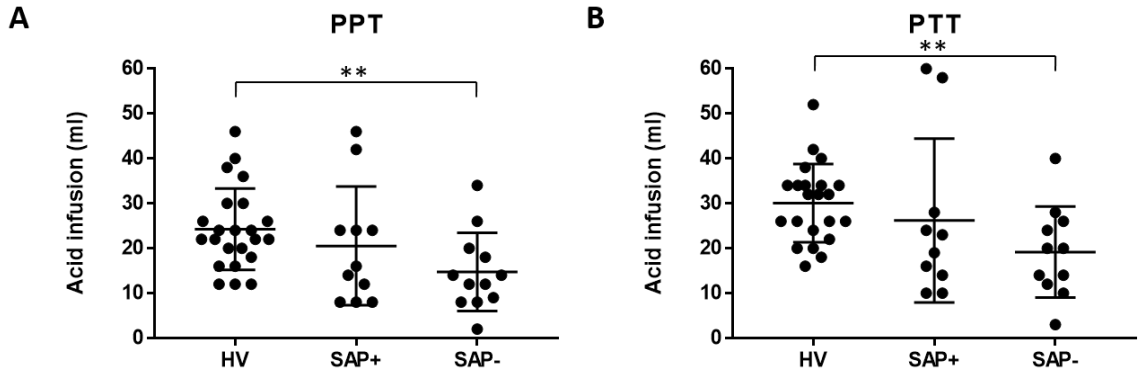

Figure 4.9 Results of esophageal chemical stimulation in HV and rGERD patients subdivided into patients with a positive SAP (SAP+) and negative SAP (SAP-). Post-hoc tests corrected for multiple testing revealed significantly lower **A)** PPT and **B)** PTT in SAP- patients compared to HV. ** p<0.01 Abbreviations: PTT=pain tolerance threshold, PPT=pain perception threshold, SAP=symptom association probability.

The rGERD cohort was also subdivided into patients with a positive and negative symptom index (SI+, n=12 and SI-, n=11, respectively). Table 4.6 shows the differences between HV and the two patient subgroups.

Table 4.6 Results of esophageal multimodal stimulation in rGERD patients subdivided into SI+ and SI- groups.

	SI +	SI -	HV	p-value$_{uncorrected}$
Temperature (°C)				
PPT	44.98 [40.18-49.4]	44.79 [42.81-47.27]	46.72 [43.8-48.65]	0.62
PTT	46.95 [42.24-51.07]	46.25 [45.31-50.96]	49.02 [46.00-51.31]	0.62
Mechanical (mL)				
PPT	17.30 [12.10-18.90]	18.10 [13.10-21.05]	19.59 [12.78-24.50]	0.24
PTT	26.20 [14.60-27.50]	18.70 [15.40-25.40]	25.00 [20.20-29.15]	0.38
Electrical (mA)				
1st perception	4.33 [3.58-11.00]	5.17 [4.17-7.33]	6.25 [4.54-9.17]	0.49
PPT	9.50 [6.75-15.50]	9.83 [7.83-15.67]	13.00 [10.00-18.21]	0.15
Chemical (mL)				
1st perception	12.00 [6.00-13.00]	7.00 [3.50-12.00]	12.00 [8.00-16.00]	0.16
PPT	20.00 [12.50-25.50]	12.00 [8.00-18.00]	22.00 [18.00-30.00]	**0.006**
PTT	23.00 [14.00-28.00]	15.00 [10.00-24.50]	32.00 [23.50-34.00]	**0.008**

Results are presented as median [25th-75th percentile]. HV: n=23, SI+: n=12, SI-: n=11. One-way ANOVA or Kruskal-Wallis test, post-hoc *t*-tests were performed with Bonferroni correction for multiple testing. SI=symptom index, PPT=pain perception threshold, PTT=pain tolerance threshold.

One-way ANOVA revealed a significant difference between HV, SI+ and SI- for the PPT (p=0.006) and PTT (p=0.008) during chemical stimulation. Post-hoc analysis revealed that SI- patients tended to have lower PPT for chemical stimulation compared to SI+, however this did not reach statistical significance (p=0.07). SI- patients showed a significantly lower PPT for chemical stimulation compared to HV (p=0.0006) (Figure 4.10A). Similar results were found for PTT; SI- patients tended to have a lower PTT for chemical stimulation than SI+ patients but this did not reach statistical significance (p=0.19). Comparing SI- patients with HV revealed a significantly lower PTT (p=0.002) (Figure 4.10B). There were no differences between HV and SI+ patients for PPT or PTT to chemical stimulation (p=0.38 and p=0.07, respectively).

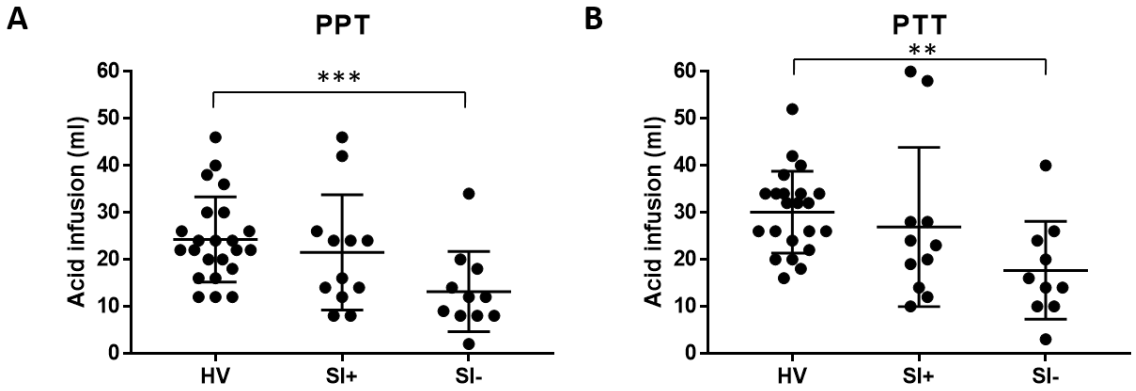

Figure 4.10 Results of esophageal chemical stimulation in HV and rGERD patients subdivided into patients with a positive symptom index (SI+) and negative symptom index (SI-). **A)** PPT and **B)** PTT to acid infusion was significantly lower in SI- patients compared to HV. ***p<0.001, **p<0.01. Abbreviations: PTT=pain tolerance threshold, PPT=pain perception threshold, SI=symptom index.

After every different stimulation modality, HV and rGERD patients were asked to indicate the referred pain area on the chest on a drawing. We could not demonstrate a difference in the location of the perceived stimulus between rGERD patients and HV. Similarly, no differences in referred pain area could be demonstrated based on the drawings of the study participants (Figure 4.11 A,B,C,D).

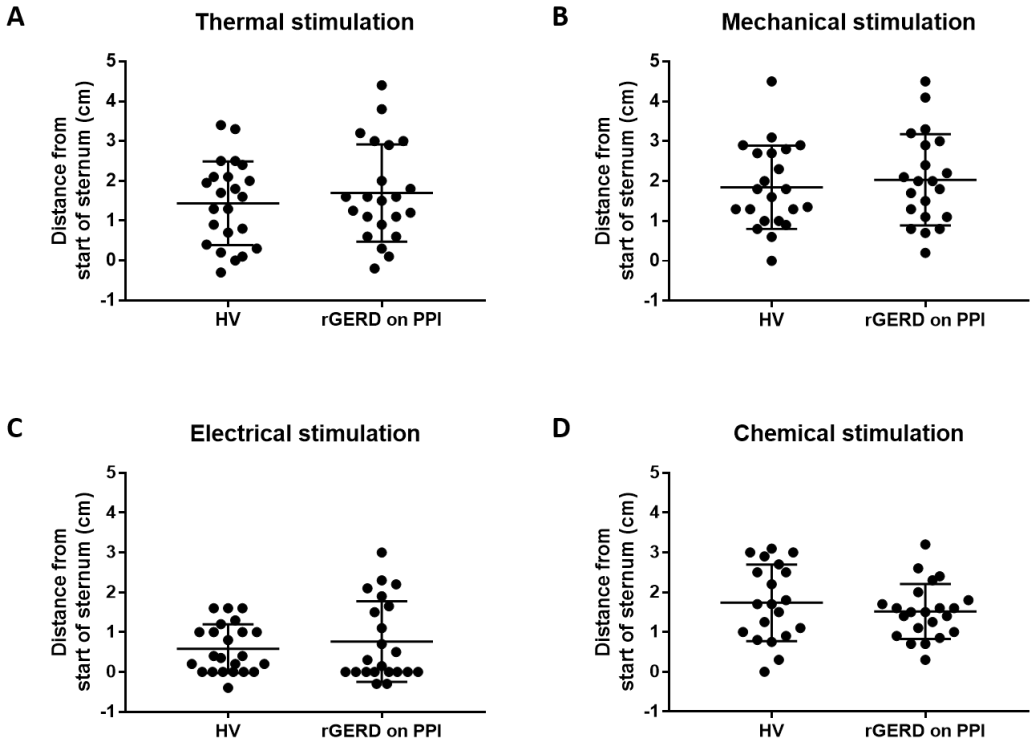

Figure 4.11 Referred pain area on the chest during multimodal esophageal stimulation. Participants were asked to indicate the location where the stimulus was perceived on a drawing. The referred pain area was similar in HV and rGERD patients in all stimulation modalities **A**, **B**, **C** and **D**.

In addition we asked the participants to indicate how they perceived the stimuli: at one specific location or rather on a broader region of the chest. When we compared the percentage of HV and rGERD patients that indicated that they perceived the stimulus at one specific location or at a broader region, we found no differences between HV and rGERD for any of the four stimulation modalities (Table 4.7).

Table 4.7 Referred pain area during multimodal stimulation in HV and rGERD on PPI. Study participants were instructed to indicate if they felt the stimulus at one specific location or at a broader region.

	Specific location		**Region**		**p-value**
	rGERD on PPI	HV	rGERD on PPI	HV	
Temperature stimulation	22.72%	39.13%	77.28%	60.87%	0.34
Mechanical stimulation	42.86%	27.27%	57.14%	72.73%	0.35
Electrical stimulation	39.13%	34.78%	60.87%	65.22%	>0.9999
Chemical stimulation	9.00%	10.00%	91.00%	90.00%	>0.9999

Before and after the multimodal stimulation test, all study participants were asked to fill out the STAI and PANAS questionnaires. Two-way ANOVA repeated measure with post hoc *t*-tests corrected for multiple testing, revealed a significant decrease in STAI scores in HV after the multimodal stimulation test compared to STAI scores before the start of the stimulation (p=0.0023) (Figure 4.12 A,B). In rGERD patients no differences in STAI scores before or after the stimulation test could be demonstrated (p=0.46). No differences in STAI-scores were found between HV and rGERD (p=0.80) (Figure 4.12A).

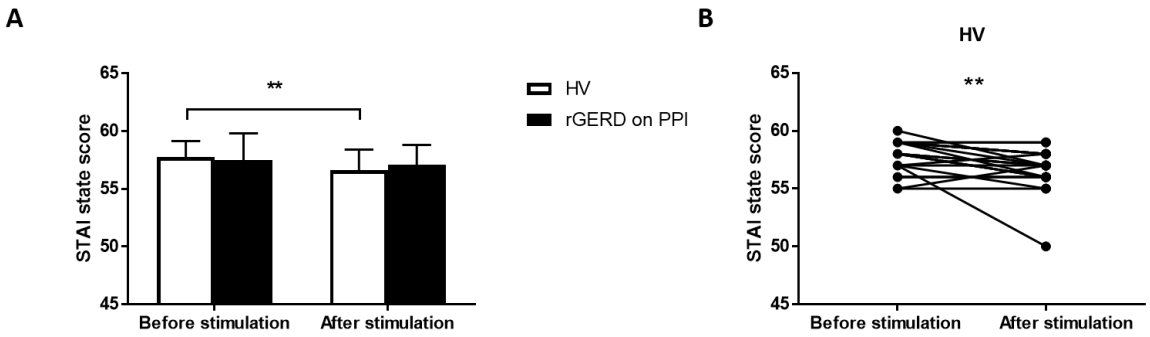

Figure 4.12 State-trait Anxiety Inventory (STAI) questionnaire results. **A)** Combined results of STAI scores before and after multimodal esophageal stimulation in HV and rGERD patients. **B)** STAI scores before and after multimodal stimulation in HV. ** p<0.01, Two-way ANOVA with Bonferroni-corrected post-hoc *t*-test.

Analysis of the positive and negative affect score questionnaire (PANAS questionnaire) showed a tendency towards lower positive affect scores in rGERD patients compared to HV. Post-hoc analysis showed that positive affect scores before the start of the multimodal stimulation test were lower in rGERD patients compared to HV (23.00 [19.25-26.50] vs. 27.50 [21.25-32.75], p=0.03), but significance was lost after Bonferroni correction. Similarly, positive affect scores tended to be lower in rGERD patients in comparison with HV after the stimulation test (23.00 [18.25-29.00] vs. 26.00 [22.00-34.00], p=0.06) but this difference did not reach statistical significance (Figure 4.13). No alterations in positive affect scores were found before and after the stimulation in both HV and rGERD patients (Figure 4.13).

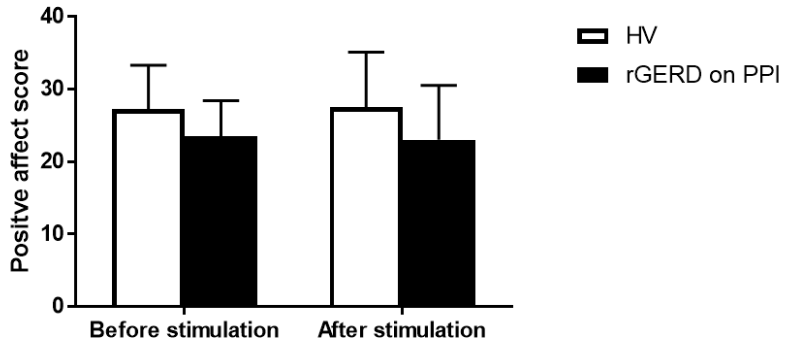

Figure 4.13 Positive affect scores in HV and rGERD patients before and after the multimodal esophageal stimulation test. rGERD patients tended to have lower positive affect scores in comparison with HV before and after the stimulation test. Two-way ANOVA with Bonferroni-corrected post-hoc *t*-test.

Negative affect scores before the start of the multimodal stimulation test were significantly higher in rGERD patients compared to HV (14.50 [13.00-17.50] vs. 12.00 [10.00-13.00], p=0.0002) (Figure 4.14A). Furthermore, we found a significant decrease in negative affect scores of rGERD patients before and after the stimulation test (14.50 [13.00-17.50] vs. 11.00 [10.00-14.00], p=0.0004) (Figure 4.14B). On the contrary, no differences were present in negative affect scores of HV before and after the multimodal stimulation (Figure 4.14A).

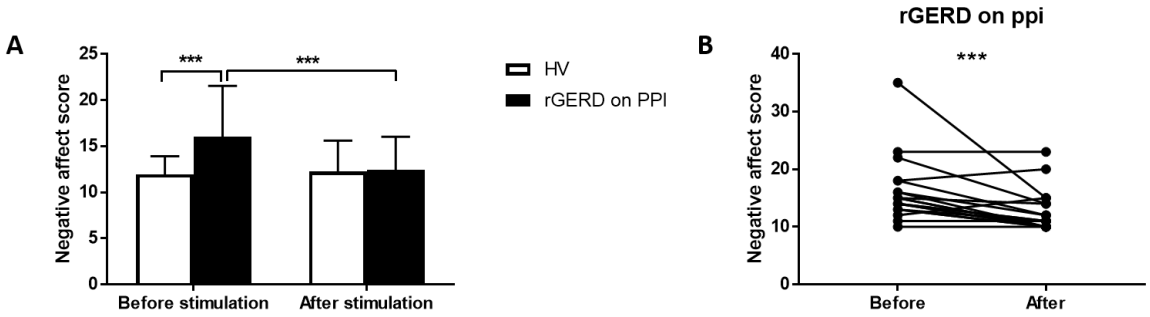

Figure 4.14 Negative affect scores in HV and rGERD patients before and after the multimodal esophageal stimulation test. **A)** Negative affect scores of rGERD patients before the stimulation test were significantly higher compared to HV, and **B)** significantly decreased after the stimulation test. No differences were demonstrated in HV. Two-way ANOVA with Bonferroni corrected post-hoc *t*-tests. ***p<0.001.

4.3.3 Changes in esophageal epithelial integrity underlying alteration in esophageal sensitivity

Ussing chamber experiments were performed to evaluate esophageal epithelial integrity both in rGERD patients on PPI as well as in HV (rGERD n=17, 8m/9f mean age 40 years, range [19-62], HV: n=21, 11m/10f, mean age 25 years, range [19-48]). Patients were significantly older than HV, but no correlations were found between age and TEER and passage values.

Although median passage of fluorescein in the distal esophagus of rGERD patients was numerically higher compared to HV, this did not reach statistical significance (31.28pmol [22.59-54.55] vs. 25.18pmol [16.42-59.49], p=0.40, Figure 4.15A). Values of distal TEER in rGERD patients were similar to that in HV (205.9Ω.cm^2 [165.9-230.4] vs. 190.0Ω.cm^2 [135.5-214.9], p=0.60) (Figure 4.15B).

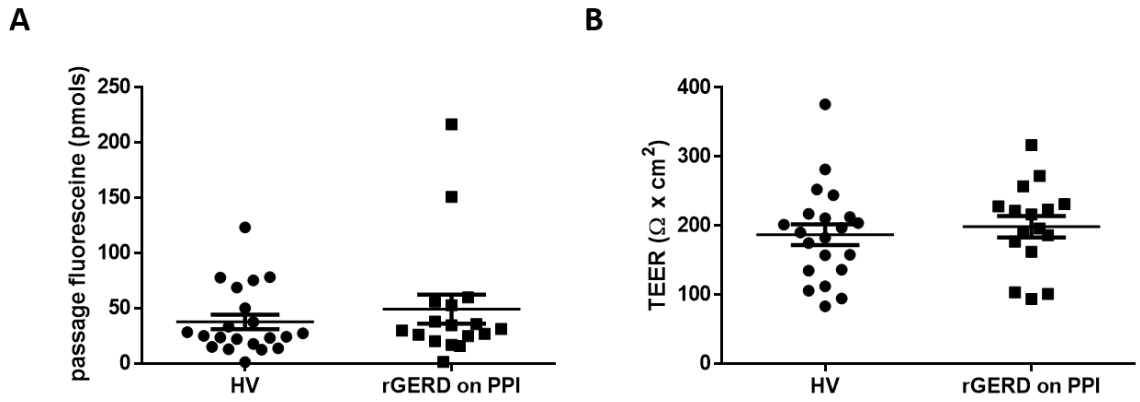

Figure 4.15 Esophageal epithelial integrity in the distal esophagus evaluated by the Ussing chamber technique. HV: n=21, rGERD on PPI: n=17. **A)** Esophageal integrity assessed by esophageal permeability, passage of fluorescein, **B)** Esophageal integrity assessed by esophageal TEER. Abbreviations: TEER= transepithelial electrical resistance.

Similar results were observed when analyses were performed on biopsies obtained from the proximal esophagus (Figure 4.16A). Median passage of fluorescein in rGERD patients on PPI was similar compared to HV (33.17pmol [15.04-61.82] vs. 31.84pmol [17.97-73.45], p=0.53). We found no significant differences in TEER values of biopsies taken in the proximal esophagus of rGERD patients compared to HV (163.3Ω.cm^2 [141.8-264.1] vs. 165.3Ω.cm^2 [116.0-207.5], p=0.2455, Figure 4.16B).

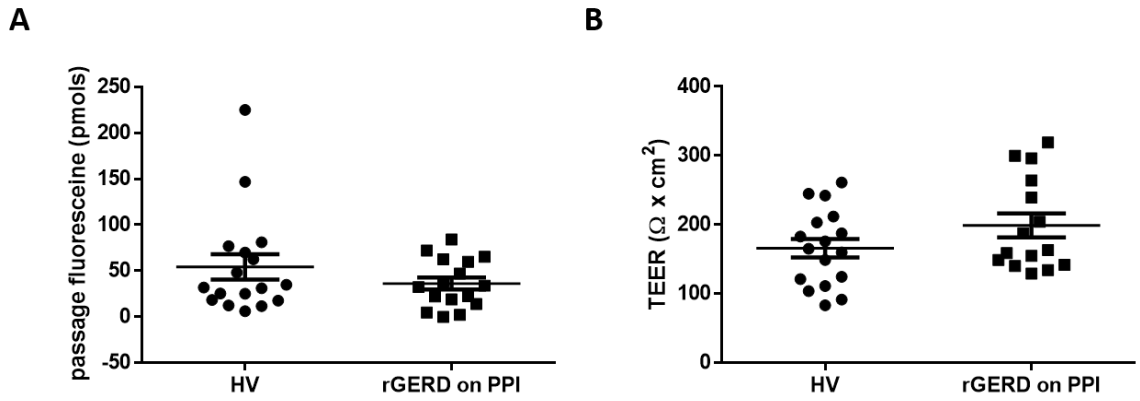

Figure 4.16 Esophageal epithelial integrity in the proximal esophagus evaluated by the Ussing chamber technique. HV: n=17, rGERD on PPI: n=16. **A)** Esophageal integrity assessed by esophageal permeability, passage of fluorescein, **B)** Esophageal integrity assessed by esophageal TEER. Abbreviations: TEER= Transepithelial electrical resistance.

Thereafter, data of esophageal epithelial integrity measurements (TEER and passage of fluorescein), reflux parameters and esophageal sensitivity tests were pooled for HV and rGERD patients to calculate the Pearson's or Spearman's correlation coefficient. When esophageal sensitivity measures were correlated with TEER or passage of fluorescein we did not find any associations between the outcome of the multimodal esophageal sensitivity tests and epithelial integrity measurements.

Linking the results of esophageal sensitivity measures to reflux parameters obtained by 24 hour MII-pH revealed no significant correlations. Correlating esophageal epithelial integrity measures with reflux parameters also showed no significant correlations.

Impedance baseline values in the upright position were assessed as an *in vivo* parameter of esophageal integrity. Baseline impedance values at 3cm were similar in HV and rGERD patients on PPI therapy (2008Ω [1435-2417] vs. 2186Ω [1287-2538], p=0.99]. Baseline impedance values at 5cm and 15cm were lower in HV compared to rGERD on PPI (5cm: 1503Ω [1371-1802] vs. 2075Ω [1494-2974], p=0.01; 15cm: 2123Ω [1475-2612] vs. 2954Ω [2376-3258], p=0.002) (Figure 4.17 A,B,C). Exclusion of the HV with an abnormal number of total reflux

events (n=8) did not have an influence on the outcome of the comparison of impedance baseline values in HV and rGERD patients.

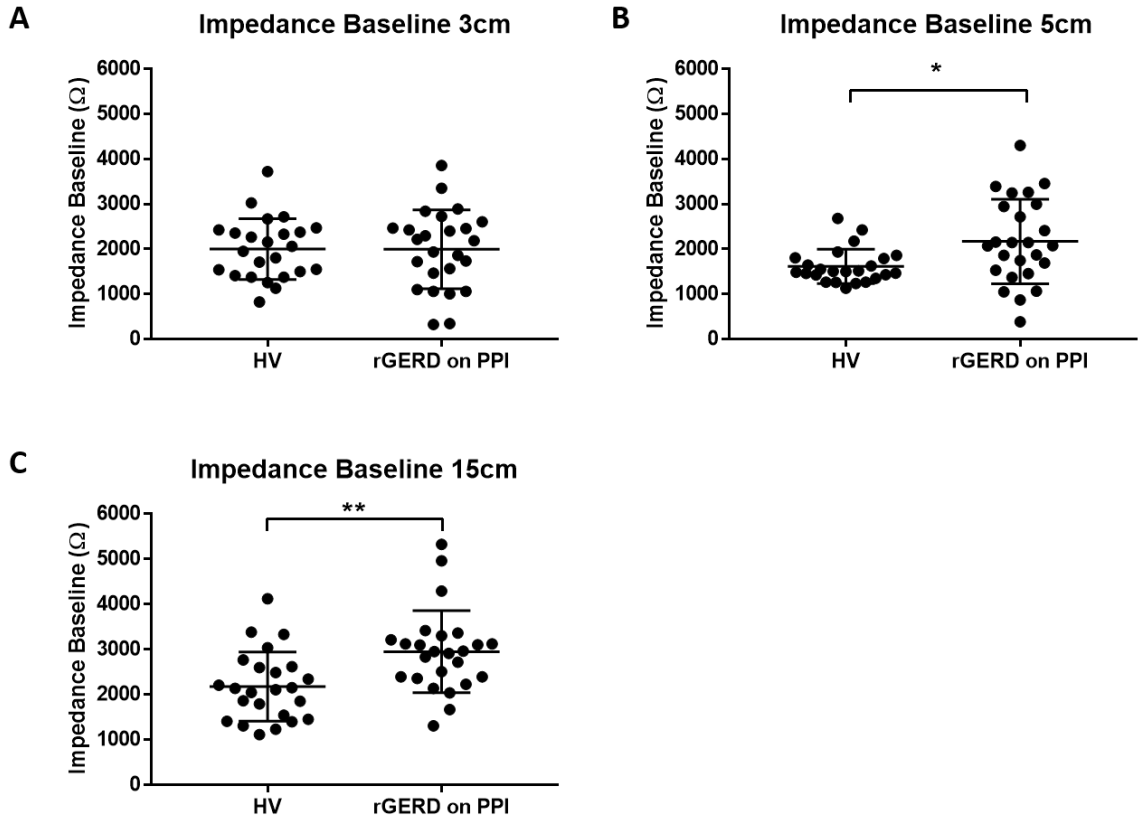

Figure 4.17 *In vivo* evaluation of epithelial integrity in HV and rGERD on PPI by impedance baseline measurements. **A)** Measurements of impedance baseline values at 3cm above the LES were similar in HV and rGERD on PPI. **B)** Impedance baseline values at 5cm above the LES were lower in HV compared to rGERD on PPI **C)** Impedance baseline values assessed at 15cm above the LES were significantly lower in HV in comparison with rGERD. *p<0.05, ** p<0.01. Abbreviations: LES=lower esophageal sphincter.

Western blot analysis was performed to assess changes is protein expression of acid sensitive receptors in distal esophageal biopsies of 7 rGERD patients on PPI and 7 HV. Vinculin was used as housekeeping protein to normalize results. No increases in protein expression of δENaC (0.82-fold, p=0.33), PAR-2 (1.15-fold, p=0.19) and ASIC3 (0.93-fold, p=0.83) were found in rGERD patients (Figure 4.18 A,B,C,D). Western blot analysis of TRPV1 failed due to lack of specificity of both antibodies used to detect TRPV1 in esophageal biopsies.

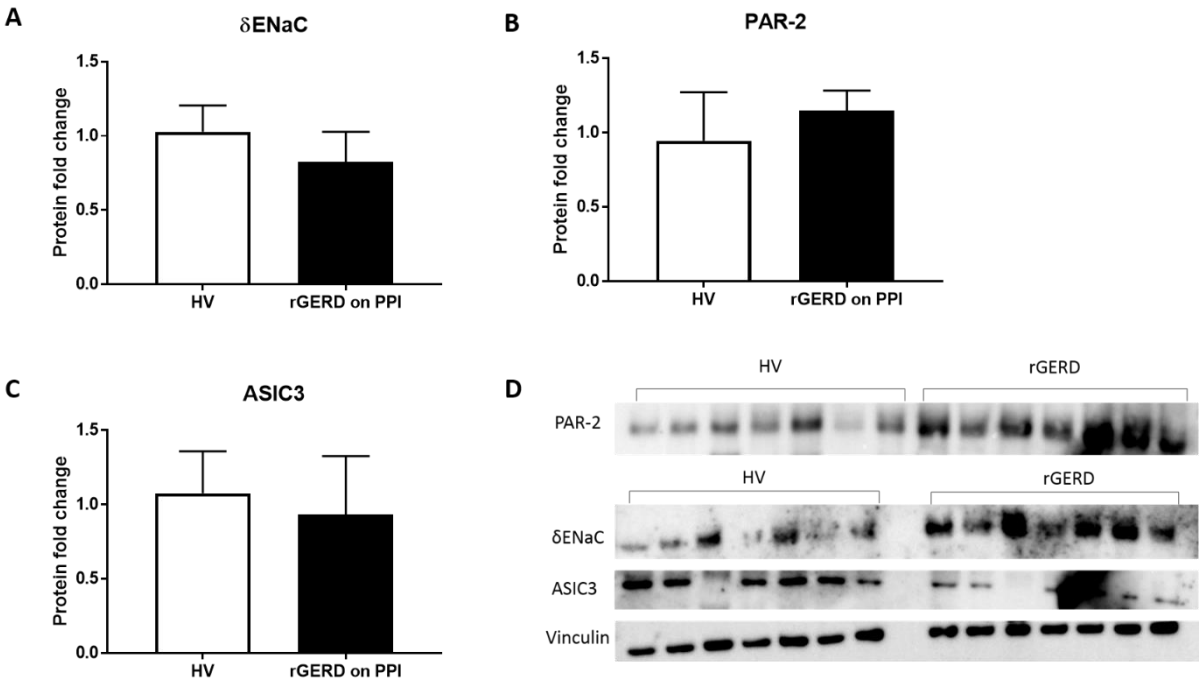

Figure 4.18 Protein expression of acid sensitive receptors in esophageal mucosa. Vinculin was used as a housekeeping protein to normalize results and protein fold change was determined relative to the mean value of the control group. Data are presented as median and interquartile ranges; n=7 for both groups. **A)** Protein expression of δENaC was similar in distal esophageal biopsies of HV and rGERD on PPI (0.82-fold, p=0.33). **B)** No differences were found in protein expression of PAR-2 in esophageal biopsies of HV and rGERD on PPI (1.15-fold, p=0.19). **C)** Protein expression of ASIC3 in esophageal mucosa of HV and rGERD on PPI did not show any differences (0.93-fold, p=0.83). **D)** Representative Western blot of 7 rGERD patients and 7 HV. Abbreviations: δENaC=delta subunit of the epithelial sodium channel, PAR-2=protease activated receptor-2, ASIC3= acid sensitive ion channel 3.

Figure 4.19 shows the result of the immunofluorescent stainings performed in distal esophageal biopsy specimens of one HV and one rGERD on PPI. Representative confocal images were taken to show the presence of PAR-2 and δENaC in esophageal epithelium. No quantification of these acid sensitive receptors has been performed thus far. Demonstration of the presence of ASIC3 and TRPV1 in distal esophageal epithelium failed due to lack of specificity of the antibodies used for immunofluorescent staining.

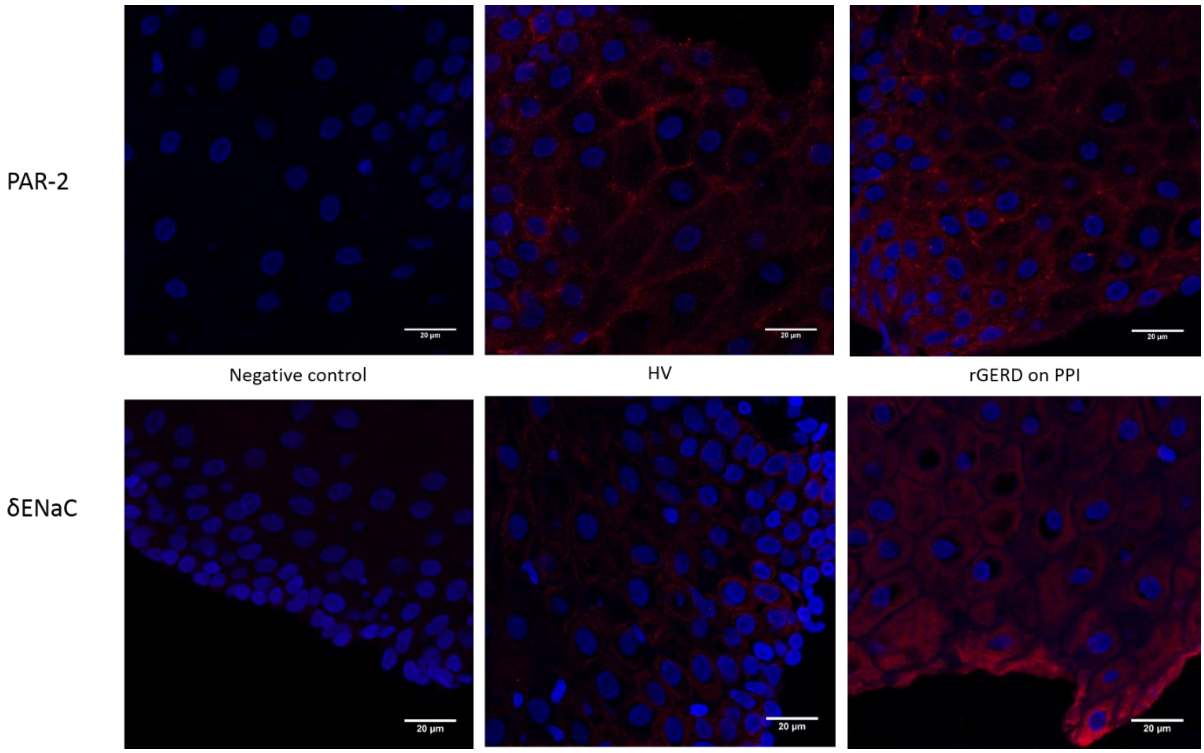

Figure 4.19 Representative confocal immunofluorescent images of esophageal epithelium of a healthy control and rGERD patient stained for protease activated receptor 2 (PAR-2) and the delta subunit of the epithelial sodium channel (δENaC), both in red.

4.4 Discussion

It remains largely unclear if acid reflux is playing a notable role in the generation of refractory GERD symptoms. The aim of this study was to characterize patients with refractory GERD symptoms on PPI therapy to identify which factors are involved in symptom generation and symptom perception.

As a first step in this project we compared 24 hour MII-pH monitoring between healthy controls and rGERD patients on PPI therapy. The results of the reflux monitoring indicate that acid exposure in the group of patients on PPI is normalized by acid suppressive therapy. Furthermore, the number of acid reflux events is similar in HV and rGERD patients, but the number of non-acid reflux events was significantly higher in rGERD compared to HV. This confirms the results of earlier studies by Vela *et al.* where a shift was found from acid to non-acid reflux in patients on PPI therapy (149). Volume exposure and the number of proximal reflux events was higher in our group of rGERD patients, suggesting that not only the number of reflux events but also the volume of the refluxate can play an important role in rGERD pathogenesis (56, 150, 151). This hypothesis was already proposed by Tsoukali *et al.* who stated that esophageal distention by increased volume reflux can be a possible mechanism of symptom generation. Furthermore, the authors suggested that mixed weakly acidic reflux events, containing gas, might produce increased distention of the proximal esophagus and are more likely to be perceived than pure liquid reflux events (151). Although the percentage of mixed reflux events was not significantly different in rGERD patients compared to HV, the distribution of mixed versus liquid reflux was different in rGERD patients compared to HV. HV had a significantly lower amount of mixed reflux events than liquid reflux events while in rGERD patients the amount of liquid and mixed reflux events was similar. However, as described below, based on our esophageal sensitivity tests we could not confirm that rGERD patients indeed have an increased sensitivity to balloon distention. Our patient cohort did not show a higher sensitivity to increasing balloon volume compared to HV. The fact that balloon distention during multimodal stimulation is performed in the distal esophagus rather than in the proximal esophagus could be a possible explanation.

Since our rGERD patient cohort was older than the healthy control group we investigated the effect of age on reflux parameters. When age was correlated to the reflux parameters of interest we found no significant associations. For the multimodal stimulation we found a

negative association between increasing age and the pain tolerance threshold to acid infusion, but significance was lost after correction for multiple testing. In literature, data on the effects of age and gender are somewhat ambiguous (152). Richter *et al.* postulated that age does not have a major effect on pH parameters (152). In 1993 Fass and colleagues published an article in which the age-difference in 24 hour esophageal pH-monitoring of normal subjects was described. Patients younger than 65 and above 65 years were compared and it was shown that the older group had higher acid exposure time, indicating that age affects reflux parameters (153). The maximal age of our participants was 66 years (n=1), so based on the findings of Fass and colleagues we argue that age in our cohort of HV and rGERD on PPI did not have an impact on the outcome of our results. In 2011, Becher *et al.* performed a systematic review of the literature to assess how age affects the prevalence of GERD and concluded that epidemiological studies do not clearly show a higher prevalence of GERD symptoms with increasing age. However in GERD patients, ageing is associated with more severe acid reflux and esophagitis; despite this, symptoms associated with GERD become less severe and more nonspecific with increasing age (154) .

Based on the cut-off values published by Zerbib and colleagues (20), 'silent reflux' was present in 8 out of 23 HV. Our healthy controls did not show pathological acid exposure but in these 8 HV an abnormal number of total reflux events (>53) was present. The cut-off values for reflux parameters remain a point of discussion. Recently, Roman *et al.* published a review article that stated that presence of esophagitis grade C or D, peptic stricture, proven Barrett's esophagus and esophageal acid exposure greater than 6% are sufficient to define pathological GER (22). Currently, there is not sufficient evidence to evaluate the exact role of the number of reflux events. However, the total number of reflux episodes and impedance baseline measurements are proposed to be an exploratory tool for further research and can be included amongst a range of additional tests in order confirm GERD, in case of borderline abnormal AET (22). Based on this consensus, the fact that 8 HV in our cohort had an increased number of total reflux events will not have major clinical implications and therefore it was not considered problematic to include these HV in our cohort since acid exposure time was far below the cut-off value of 6%. Also the number of reflux episodes in these 8 subjects did not exceed the number of 80 events which was considered to be a clearly high number of reflux events by the consensus group.

Concerning the esophageal sensitivity tests, we were not able to replicate the data previously shown by our group. Based on the sensitivity tests performed with the multimodal stimulation protocol, we were only able to show an altered sensitivity to pain perception threshold during electrical stimulation and altered sensitivity thresholds during acid infusion in rGERD patients compared to healthy controls. No differences in thresholds for the thermal and mechanical stimulation could be demonstrated in our larger cohort. As mentioned above, these results are in contrast to an earlier study in our group. A plausible explanation could be the rather small sample size in the previous study.

In the current study we included 23 rGERD patients and we were able to show robust differences in sensitivity to acid infusion and a lower sensitivity threshold for pain perception during electrical stimulation. When we subdivided our patients into patients with a positive and negative symptom association (SAP+/- and SI+/-), we found that patients with a negative symptom association systematically have lower sensitivity thresholds. In SI- negative patients especially the sensitivity to acid infusion was higher compared to HV and SI+ patients. In contrast to our current findings, available literature indicates that patients with a negative symptom association are likely to have functional heartburn, it is assumed that their symptoms are not related to GERD.

Our results suggest the opposite, as patients with a negative symptom association (SAP and SI) were more sensitive to multimodal esophageal stimulation. A first reason for this apparently opposing result could be the relatively small number of patients in each group. Therefore, caution is warranted to draw conclusions based on these subgroups. Secondly, artificial stimulations to assess acid sensitivity do not fully mimic actual reflux events. During our modified Bernstein test, a constant instillation of an acid solution is performed while reflux events are usually a short episodic acid stimulation. Furthermore, the direction is different, reflux occurs in retrograde direction while during artificial stimulation there is an anterograde flow of acid. The volume is usually smaller in real reflux events compared to the volume of artificial acid infusion. Finally, the composition of the refluxate (including pepsin, bile salts and air) differs from the artificial HCl solution. This discordance has already been addressed Jung by *et al.* (155).

In addition, the categorization of patients into reflux hypersensitivity and functional heartburn may be incorrect when based on the results of a 24 hour MII-pH measurement, which is

common practice in many centers. Based on day to day variability in acid exposure, it is conceivable that patients could be subdivided into another subgroup of the GERD spectrum when the measurement was performed on a different day. Studies that have performed reflux monitoring that lasted more than 24 hour, indicate that prolonged recording time could increase sensitivity of reflux detection and symptom events for symptom association analysis (22). Gyawali *et al.* demonstrated that extending recording time to 48 hours with a wireless pH monitoring system (BRAVO pH capsule system) increases the likelihood of detecting reflux disease in patients undergoing symptom evaluation. It has been stated that benefits of prolonged recording time are most evident for patients with atypical symptoms of GERD and for more accurately establishing reflux symptom association analysis (156, 157).

Since psychosocial factors are known to be involved in rGERD, study participants were asked to fill out the PANAS and STAI questionnaires before and after the multimodal stimulation protocol. We found significantly higher negative affect scores in our rGERD patient cohort compared to HV, indicating that patients in our study were more anxious at the start of the stimulation test than HV. This could have an impact on the outcome of the stimulation test since it has been shown that a negative emotional status can aggravate the perception of pain stimuli (158). Furthermore, our group has previously shown that affective disorders, somatization and body awareness are significantly and independently associated with positive symptom association during 24 hour MII-pH monitoring in patients with GERD symptoms (159). In accordance with this study, our data further suggest that psychosocial factors might contribute to reflux sensitivity in patients with GERD.

Alterations in esophageal sensitivity could be caused by an impaired esophageal barrier function (52, 78). In our *in vitro* measurements of esophageal integrity, we observed a substantial overlap between HV and our patient population for measurements of TEER and passage of fluorescein. No significant differences in TEER and permeability were demonstrated in our cohort of rGERD compared to HV. This can be partially attributed to a rather heterogeneous patient group which is inherent to the rGERD patient population. However, the fact that our patients were on a double dose of PPI therapy probably was the main reason for the absence of an impaired esophageal epithelial integrity. It can be argued that impaired mucosal integrity does not seem to play a major role in persisting symptoms in refractory GERD patients on a double dose of PPI.

The *in vivo* assessment of epithelial integrity was performed by impedance baseline measurement at 3, 5 and 15cm above the LES. Impedance baseline values were measured in the upright position since recent results from our group indicate that measurement in the upright position are more useful in comparison to the recumbent position to discriminate between subgroups of rGERD (160). When comparing impedance baseline values in HV and rGERD patients we found a significantly lower impedance baseline at 5 and 15cm above the LES in HV compared to rGERD on PPI. Only recently, Pardon *et al.* compared the impedance baseline values of rGERD patients and HV on PPI and showed that impedance baseline values at 15cm above the LES were similar in rGERD patients on PPI and HV on PPI (160). However, in our study, HV were not on acid suppressive therapy while rGERD patient were on a double dose of PPI which could attribute to the differences observed in impedance baseline values which has also been demonstrated in a recently published paper by Pauwels *et al.* (160, 161). Furthermore, impedance baseline values in our cohort of healthy controls were relatively low in comparison with impedance values of healthy controls reported by other groups (162-164). The reason for this discrepancy is not clear since we found no correlation between acid exposure time and baseline impedance values at 3cm, 5cm and 15 cm. The large variability in impedance values in our cohort of HV can be a possible explanation.

Preliminary data on presence and distribution of the acid sensitive receptors PAR-2, δENaC, and ASIC3 were shown in this chapter. Based on Western blot analysis we were not able to show a difference in protein expression levels of these acid sensitive receptors in esophageal biopsies in HV and rGERD patients on PPI. We cannot exclude that long term treatment with PPIs by itself has an influence on the expression of acid sensitive receptors. It has been documented that PAR-2 expression was induced by exposure to acid and weakly acidic solutions making it plausible that PPI treatment can have an impact on PAR-2 expression (165). However, the actual effect of PPI treatment on the expression of acid sensitive receptors is unclear since most studies investigating the involvement of these receptors are performed after discontinuation of acid suppressive therapy. One of the limitations of this study was the fact that Western blot analysis was performed in rather small numbers. In future projects we will focus on increasing the sample sizes and the outcome of quantification of Western blot analysis should be confirmed with PCR analysis since we acknowledge that it is disputable to draw robust conclusions based on Western blot analysis alone. Furthermore, additional

experiments are needed to optimize conditions to show the presence of TRPV1 in esophageal epithelium. In the current study, the antibodies used for TRPV1 did not show adequate specificity, therefore we were unable to draw conclusions about the role of TRPV1 in rGERD patients.

In conclusion, the overall objective of this study was to investigate why rGERD patients continue to experience symptoms of GERD while on a double dose of PPIs. Based on our results we conclude that i) altered reflux parameters besides acid reflux are important: the number of non-acid reflux events and the proximal extent of reflux episodes was higher compared to healthy volunteers, suggesting that volume reflux and proximal reflux events are more likely to be involved in symptom generation of rGERD. ii) We confirmed the presence of esophageal hypersensitivity in rGERD, mainly to acid infusion and to a lesser extent to electrical stimulation as shown by the multimodal esophageal stimulation protocol. iii) Impaired esophageal epithelial integrity did not seem to be involved in increased esophageal hypersensitivity in rGERD patients on a double dose PPI treatment. iv) We demonstrated that in rGERD patients, feelings of anxiety and negative affect are more present compared to HV and are likely to play a role in an altered perception of GERD symptoms.

CHAPTER 5

FAILURE OF ANTI-NOCICEPTIVE PATHWAYS

5 Failure of anti-nociceptive pathways

5.1 General introduction

The GI tract has an extensive sensory innervation: signals from the periphery are conveyed to the central nervous system (CNS) via two distinct afferent systems: i) the vagal nerve and ii) the spinal nerves, both of which are part of the autonomic nervous system. Via these sensory afferents, the brain and the GI tract are highly integrated and communicate in a bidirectional way. This system is generally referred to as the brain-gut axis (166). When homeostatic information from the GI tract is transmitted to CNS, gut sensations are mediated and, if needed, integrated with an appropriate autonomic or behavioral response (*e.g.* regulation of food intake) (167). In physiological conditions, only gut-brain signals that require a behavioral response are consciously perceived. However, in pathological conditions (*e.g.* peripheral or central sensitization), an aberrant, heightened perception of these gut-brain signals can develop and this phenomenon is termed visceral hypersensitivity (65). A complex interplay exists between neurotransmitters and neuroendocrine factors that are part of the sensing mechanisms of the GI tract. These GI sensing mechanisms can be altered by a wide range of mediators. Indeed, it has been previously demonstrated that plasticity in nociceptive pathways exists and injury or inflammation is able to change the transduction properties of these pathways and make them hyper-responsive to a given stimulus (120). In this regard, it has been shown that esophageal mechanosensitivity can be influenced by a number of chemical mediators such as acid present in gastric contents during the occurrence of GER. Furthermore, previously mechanical insensitive afferents can develop mechanosensitivity during inflammation and an enormous variety of chemical mediators such as serotonin, prostaglandins, adenosine, histamine, proteases and many others are involved in this sensitization process (65, 167, 168). Visceral hypersensitivity is considered to be a hallmark of functional GI disorders and also in GERD it has been postulated to be a potential underlying mechanism of symptoms refractory to acid suppressive therapy (169, 170). To further unravel the nociceptive pathways of the brain-gut axis that are involved in esophageal hypersensitivity in general and heartburn perception more specifically, we investigated three candidate neurotransmitter systems that may be involved in the central processing and modulation of esophageal afferent signals: i) the endogenous opioid system, ii) the serotonin system and iii) the dopamine system.

5.2 Blocking the endogenous opioid system

5.2.1 Introduction

Endogenous opioid neurotransmission plays a central role in many physiological processes including respiration, analgesia and pain signaling (171). In addition, opioids are a key candidate in endogenous anti-nociceptive pathways. They mediate their effects by acting on δ-, κ- and μ-receptors which are found throughout the enteric nervous system and CNS and are known to influence GI motility and sensation (172-174). Opioid receptors are present in the submucosa and on neurons in the myenteric plexus of the stomach, the small intestine and the colon (172, 174). Animal studies in the early eighties have already shown the presence of opioid receptors in the esophageal body and the LES, however the distribution of these receptors in the human esophagus is not completely elucidated (175, 176).

Brain imaging studies have demonstrated the presence of μ-opioid receptors in brain areas which are involved in nociception and regulation of GI function (177-179). Furthermore, positron emission tomography (PET) studies using radioligands (*e.g.* [^{11}C]carfentanil) selective for the μ-opioid receptor revealed the release of endogenous opioids during sustained somatic pain in disorders such as fibromyalgia and central neuropathic pain (179-181). All together, these findings suggest that alterations in the endogenous opioid neurotransmission system could be a potential underlying mechanism in various pain disorders (179). Although a role for endogenous opioids during somatic pain has been well established, their involvement in functional GI disorders or visceral pain remains unclear.

In 2006, Staahl *et al.* investigated the anti-nociceptive properties of exogenously administered opioids in the esophagus. Esophageal sensitivity was assessed by a multimodal pain model after administration of morphine and oxycodone, two central μ-opioid receptor agonists. This study showed that the pain detection threshold for thermal stimulation increased by oxycodone administration, suggesting an analgesic effect of exogenously administrated opioids (182). On the contrary, when the role of endogenous instead of exogenous opioids in visceral pain was investigated, Ly and colleagues could not demonstrate a difference in the release of endogenous opioids in the brain during non-painful and sustained painful gastric distention in HV, concluding that that there might be a differential role for endogenous opioids in somatic and visceral pain processing (183). A study performed by Geeraerts *et al.* investigated the effect of the centrally acting μ-opioid receptor antagonist naloxone on gastric sensorimotor function. Their observations suggest that endogenous opioids are involved in

the control of gastric accommodation and phasic contractility but not in the control of sensitivity to gastric distention or gastric emptying in health (184). In another study, Janssen *et al.* investigated the role of both central and peripheral μ-opioid receptor antagonism in the regulation of gastric tone and food intake by performing a gastric barostat experiment. This study confirmed earlier results that endogenous opioids play a role in gastric accommodation. Furthermore, the authors concluded that endogenous opioids mediate gastric accommodation and satiation via peripheral μ-opioid receptors rather than via central μ-opioid receptors, which might mediate opposing effects (185).

Even though the effects of opioids on the stomach, the small and large intestine have been well studied, there are only limited data on the effects of endogenous opioids on esophageal function and esophageal sensation (175). Therefore, the aim of this study was to examine the effect of the centrally acting μ-opioid receptor antagonist naloxone and the peripherally restricted μ-opioid receptor antagonist methylnaltrexone on esophageal sensitivity in a group of HV.

5.2.2 Materials and methods

5.2.2.1 Study design

Esophageal sensitivity was assessed in HV using the multimodal stimulation protocol as described in Chapter 3 (paragraph 3.3) after administration of naloxone (0.4mg IV bolus injection followed by 20µg/kg/h IV infusion), methylnaltrexone (12mg/0.6mL SC injection) or placebo (0.9% NaCl). Three sessions were scheduled for each subject with at least one week interval to be able to compare the three different conditions: i) SC injection with methylnaltrexone and placebo bolus injection and infusion, ii) SC injection with placebo and naloxone bolus injection and infusion, iii) SC injection with placebo and placebo bolus injection and infusion. Sessions were run in a single-blind way and the order of the study visits was randomized. The outline of the study design is depicted in Figure 5.1. After an overnight fast, study participants were asked to fill out the STAI and the PANAS questionnaires. Immediately after positioning of the multimodal stimulation probe a SC injection of methylnaltrexone or placebo was administered. Since plasma levels of methylnaltrexone are maximal 30 minutes after the SC injection, a waiting period of 30 minutes was included before initiation of the multimodal stimulation test. After 25 minutes an IV bolus injection of naloxone or placebo was administered followed by IV infusion of naloxone or placebo. Hereafter, the multimodal stimulation test was initiated. At the end of the stimulation, participants filled out the STAI and PANAS questionnaires a second time.

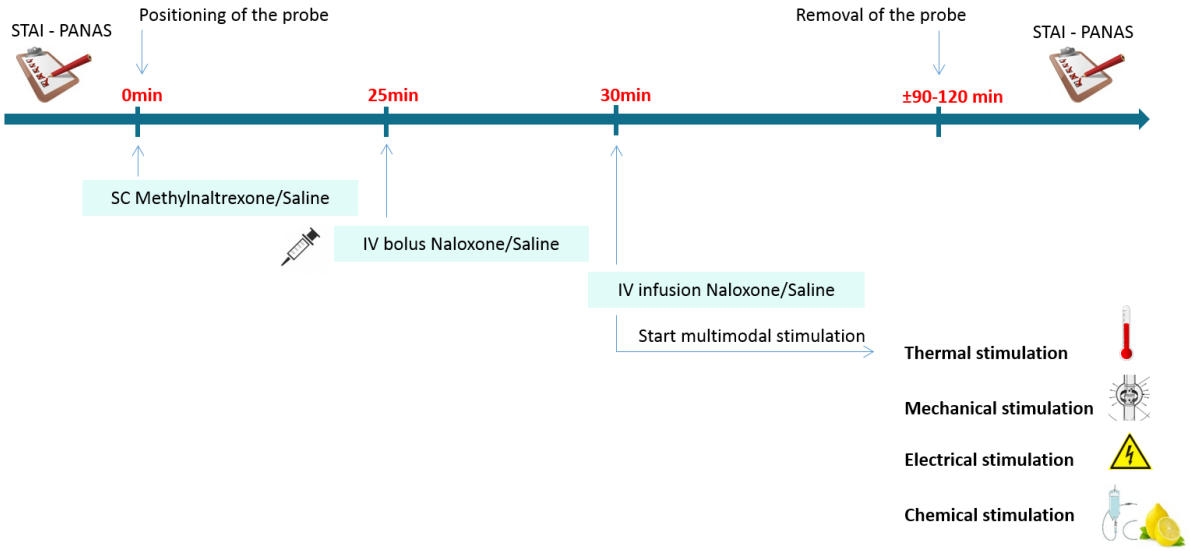

Figure 5.1 Outline of the naloxone-methylnaltrexone study. Abbreviations: STAI=State-Trait Anxiety Inventory, PANAS=Positive and Negative Affect Schedule, SC= subcutaneous, IV=intravenous.

5.2.2.2 Statistical analysis

The number of volunteers to be included was calculated with a medium comparison test (comparison of variance, GPower 3.1.9.2 software). In order to detect a 10% difference with a risk α of 5%, we needed to include 12 HV to ensure a power of 80%. Thermal, mechanical, electrical and chemical sensitivity was measured at 1st perception, pain perception threshold (PPT) and pain tolerance threshold (PTT) and were used to assess esophageal sensitivity. Statistical analysis was performed using GraphPad Prism 7.02 (GraphPad Software, Inc., La Jolla, CA USA). Esophageal sensitivity was compared between naloxone, methylnaltrexone and the placebo condition using one-way ANOVA repeated measures or a Friedman test in case of non-parametric data distribution (evaluated using the Kolmogorov-Smirnov test). Chi-squared tests were used to evaluate if there was a difference in the occurrence of a ceiling effect for the sensitivity thresholds during the stimulation tests. A p-value of 0.05 was considered to be statistically significant. P-values were corrected for multiple testing using Bonferroni correction. Data are presented as median [25th-75th percentile], unless stated otherwise.

5.2.2.3 Ethical approval

The protocol was approved by the ethics committee of the University Hospital of Leuven (approval number: S54661) and the Federal Agency for medicines and health products (EudraCT number 2012-003409-86). Furthermore, the study was registered at ClinicalTrials.gov (NCT03014843). Written informed consent was obtained from participants before inclusion in the study.

5.2.3 Results

Twelve HV (7m/5f, mean age 31 years [range 22-51]) were enrolled in this study. No side effects were reported by the participants in any of the three test conditions. When comparing the three study conditions, we found no influence of naloxone or methylnaltrexone on esophageal sensitivity to thermal stimulation. The thresholds for pain perception (PPT) and pain tolerance (PTT) were not altered after administration of the two opioid antagonists compared to placebo (PPT p=0.98, PTT p=0.91) (Figure 5.2 A,B).

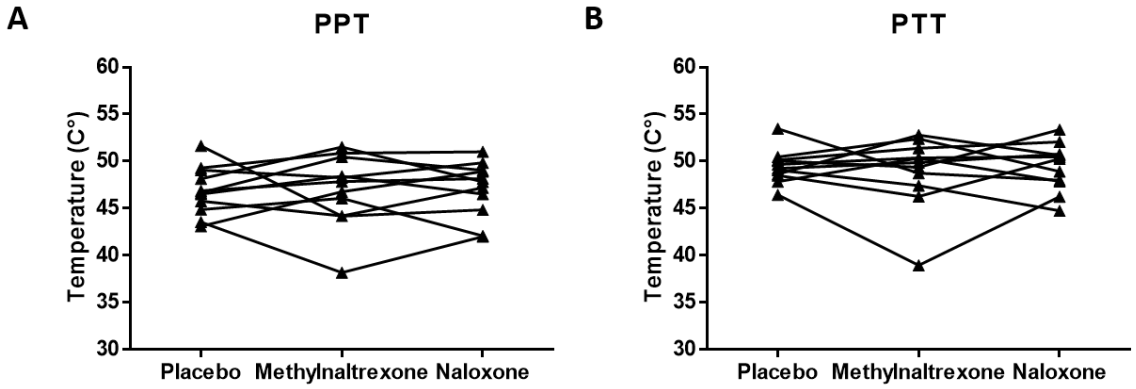

Figure 5.2 Results of esophageal temperature stimulation after administration of methylnaltrexone, naloxone or placebo. **A**, **B)** No alterations in esophageal sensitivity to thermal stimulation were seen when the three study conditions were compared. Abbreviations: PPT= pain perception threshold, PTT=pain tolerance threshold.

Similar results were found for esophageal mechanical stimulation (PPT p=0.33, PTT p=0.42). The volume at which HV reached PPT and PTT was not altered after administration of naloxone or methylnaltrexone (Figure 5.3 A,B). For the mechanical stimulation we observed an important ceiling effect: a large proportion of the participants (4/12 for PPT, 7/12 for PTT) did not reach the sensitivity thresholds at 50mL in all three study visits and were not included in the analysis. The number of participants that reached the sensitivity thresholds during mechanical stimulation was similar in the three study conditions (PPT: p=0.77, PTT: p=0.33, Chi-squared test).

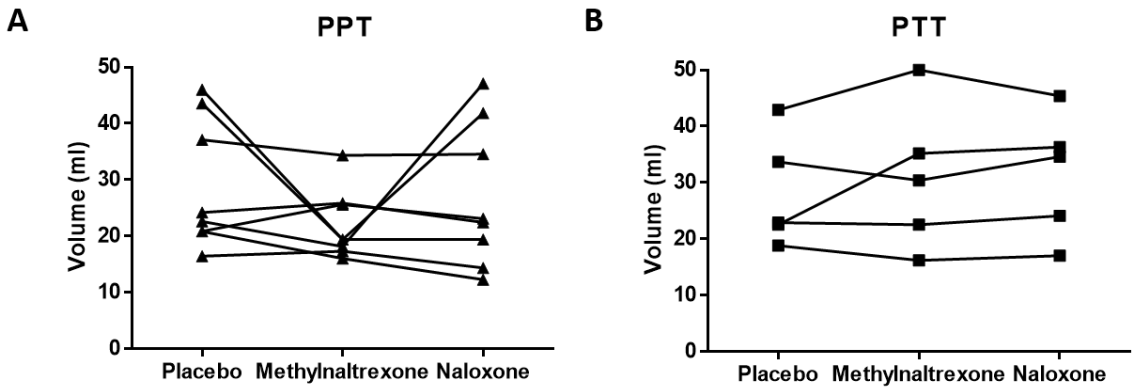

Figure 5.3 Results of esophageal mechanical stimulation after administration of methylnaltrexone, naloxone or placebo. **A**, **B)** No alterations in esophageal sensitivity to mechanical stimulation were seen when the three study conditions were compared. Abbreviations: PPT= pain perception threshold, PTT=pain tolerance threshold.

When comparing esophageal sensitivity to electrical stimulation after administration of the opioid antagonists, no differences were seen for the 1st perception threshold (p=0.95), nor for PPT (p=0.44) (Figure 5.4 A,B).

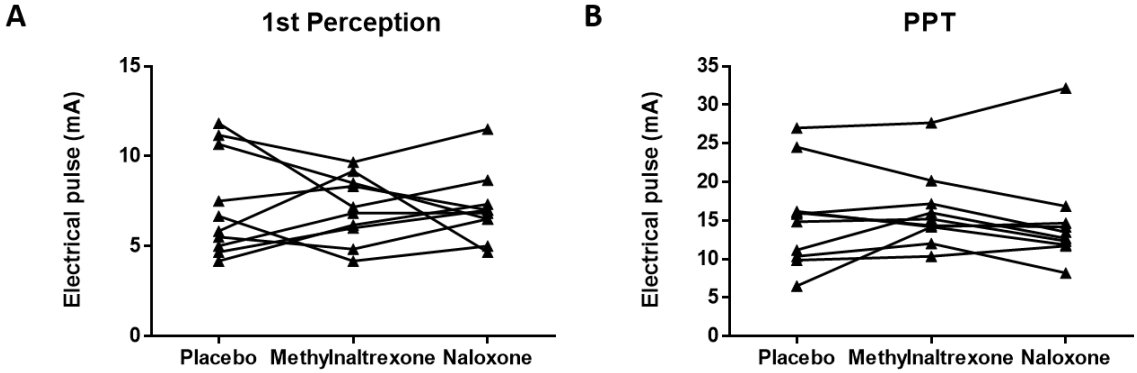

Figure 5.4 Results of esophageal electrical stimulation after administration of methylnaltrexone, naloxone or placebo. No alterations in **A)** 1st perception threshold and **B)** PPT to electrical stimulation were seen when the three study conditions were compared. Abbreviations: PPT= pain perception threshold.

Finally, as shown in Figure 5.5 sensitivity thresholds for chemical stimulation of the esophagus by infusion of an acid solution did not change after naloxone or methylnaltrexone administration compared to placebo (1st perception p=0.40, PPT p=0.92, PTT p=0.92). Similar to the mechanical stimulation, we observed a ceiling effect during the chemical stimulation. The majority of our participants, 7 out of 12 for PPT and 8 out 12 for PTT, did not reach the sensitivity thresholds after 30 minutes of acid infusion in all three study visits and were not

included in the analysis. The number of participants that reached the sensitivity thresholds was similar in the three study conditions (PPT: p=0.80, PTT: 0.89, Chi-squared test).

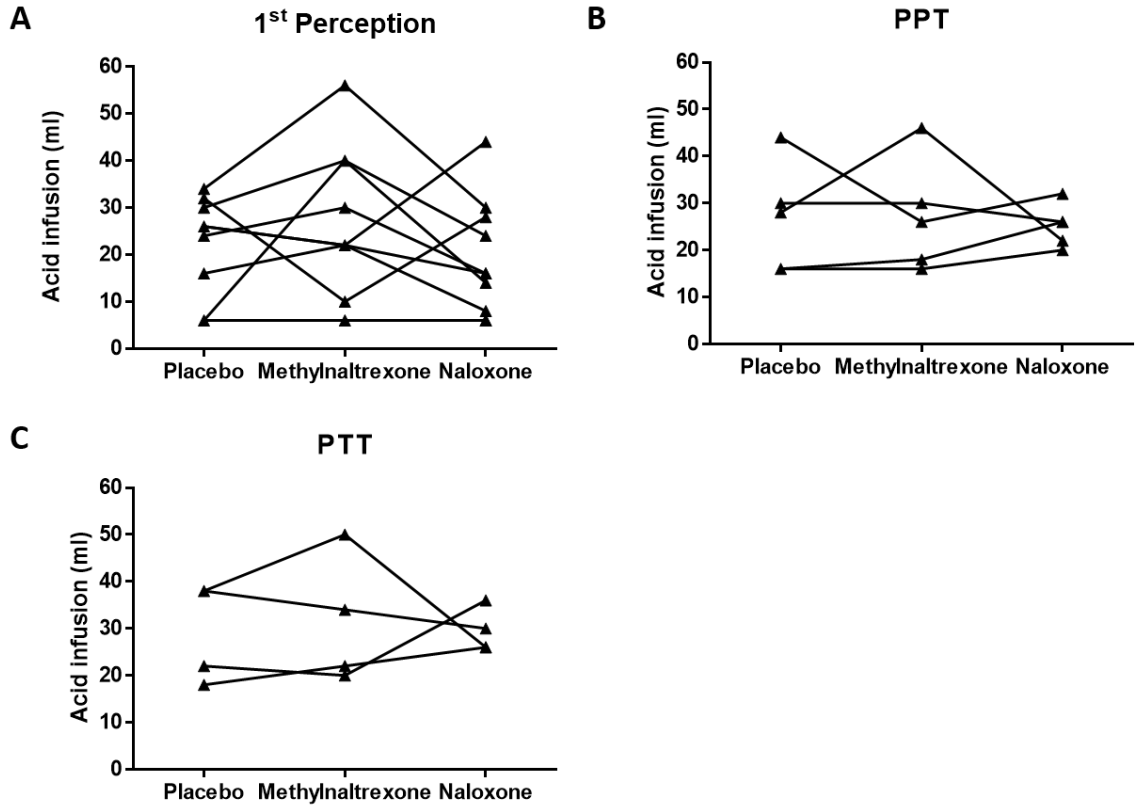

Figure 5.5 Results of esophageal chemical stimulation after administration of methylnaltrexone, naloxone or placebo. No alterations in esophageal sensitivity to chemical stimulation were seen when the three study conditions were compared. **A)** 1st perception threshold, **B)** PPT and **C)** PTT were unaffected by administration of naloxone or methylnaltrexone. Abbreviations: PPT= pain perception threshold, PTT=pain tolerance threshold.

We performed a two-way repeated measures ANOVA analysis to investigate the effect of gender on the four stimulation modalities. For thermal stimulation, thresholds for PPT and PTT (p=0.41 and p=0.85, respectively) were not different in women in comparison with men. Also thresholds for mechanical stimulation were similar in women and in men (PPT: p=0.94, PTT p=0.48). Furthermore, no gender differences were seen for electrical stimulation (1st perception: p=0.24, PPT: p=0.58) and chemical stimulation (1st perception: p=0.58, PPT: p=0.91, PTT: p=0.63).

Emotional status was assessed before and after the esophageal stimulation test by means of the STAI state and PANAS questionnaires. There were no differences in the questionnaires scores before and after the stimulations for the three different sessions. Positive and negative

affect scores (Figure 5.6 A,B) were similar in the three conditions before and after the stimulation test. Also STAI state scores were similar in all conditions (Figure 5.6C).

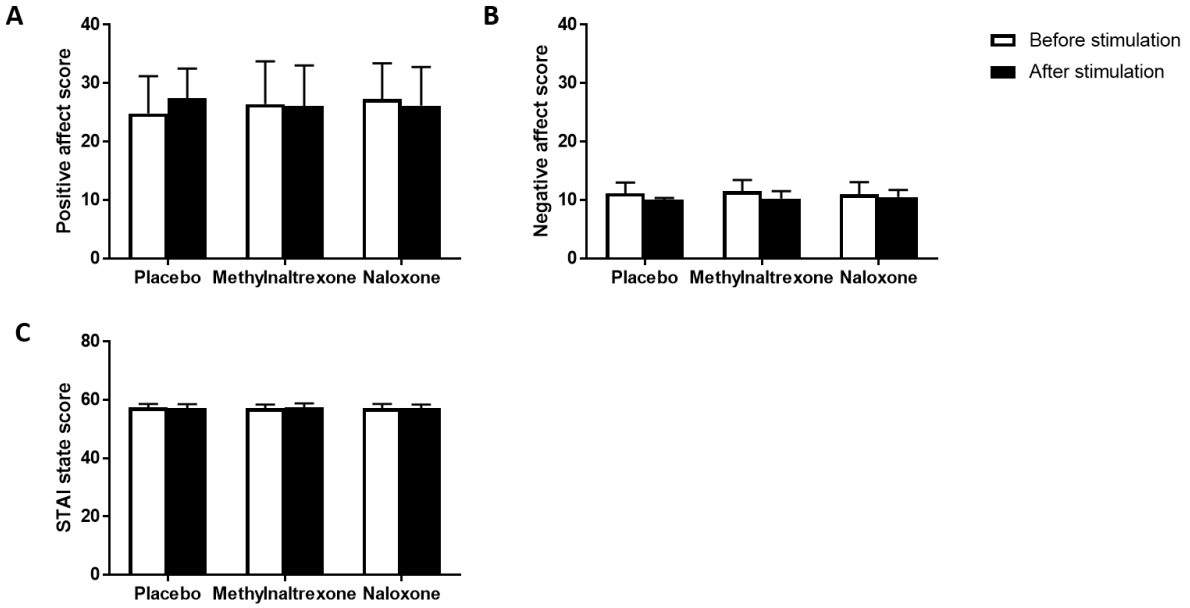

Figure 5.6 Results of **A)** Positive affect and **B)** Negative affect scores and **C)** STAI-state questionnaire scores before and after multimodal stimulation in the three study conditions. No significant differences in questionnaire scores were observed. Abbreviations: STAI=State-Trait Anxiety Inventory, PANAS=Positive and Negative Affect Schedule.

5.2.4 Discussion

It is remains unclear whether the endogenous opioid system affects the perception of visceral sensations, and more specifically esophageal sensitivity. A number of studies have investigated the effect of exogenous opioid agonists on esophageal pain perception (182, 186) and the effect of opioid antagonists on gastric sensorimotor function (184, 185). However, to our knowledge, the effect of peripheral and central opioid antagonists on esophageal sensitivity has not been studied so far. Therefore, the objective of the present study was to investigate the effect of the centrally acting μ-opioid receptor antagonist naloxone and the peripherally restricted μ-opioid receptor antagonist methylnaltrexone on sensitivity to multimodal esophageal stimulation in healthy volunteers.

In our study we found no evidence of a tonic inhibitory effect of endogenous opioid pathways on esophageal sensitivity in health since nor naloxone nor methylnaltrexone altered sensitivity to multimodal esophageal stimulation. In healthy subjects, sensitivity thresholds for thermal, mechanical, electrical and chemical stimulation remained unaltered in comparison to placebo. In addition, in the three study conditions no alterations in mood were observed after the multimodal stimulation test compared to before the start of the stimulation test.

In literature the effects of exogenous opioids on esophageal function have already previously been described. In a large retrospective study in 2015, Ratuapli *et al.* demonstrated that the chronic opioid use has an effect on esophageal motor function namely, opioid use within 24 hours of esophageal high resolution manometry (HRM) was associated with more frequent EGJ outflow obstruction and spastic peristalsis compared to when opioid use was stopped for at least 24 hours before HRM. Reports on the effects of chronic opioid use on LES pressure are conflicting, however, the majority of the studies describe an increase in LES pressure and an impairment of swallow induced LES relaxation (175).

Besides the effects on esophageal motility, only limited data are available on the effects of opioids on esophageal sensitivity. Staahl and colleagues investigated the anti-nociceptive properties of oxydocone and morphine in multimodal esophageal stimulation and demonstrated that both morphine and oxycodone attenuated pain stimuli compared to placebo (182). The results of this study confirm a potential analgesic effect of exogenously administered opioid agonists on visceral pain. Nevertheless, an important distinction must be

made between the effects of exogenous opioid agonists and endogenously released opioid peptides.

Endogenous ligands of opioid receptors, such as β-endorphins, enkephalins, dynorphins and endomorphins are synthesized and secreted by immune cells in response to a variety stressful stimuli (*e.g.* experimental pain, surgery) (187, 188). These released opioid peptides are known to activate peripheral opioid receptors and in this way they are able to modulate pain perception.

Furthermore, animal research revealed that a differential role exists for exogenously administered opioids and endogenous opioid peptides. For example, Labuz *et al.* investigated the role of opioids in the modulation of neuropathy-evoked heat and mechanical hypersensitivity in mice. The authors described that exogenously administered opioid agonists were able to attenuate heat hypersensitivity whereas endogenous opioid peptides showed no effect. A differential effect was observed for mechanical hypersensitivity, which was improved by opioid peptides. Based on their findings the authors concludes that opioid therapy for hypersensitivity requires careful tailoring according to the opioid type and modality of pain resulting from neuropathy (188, 189). The above mentioned studies investigated the effect of opioids in somatic pain sensation. The involvement of endogenous opioid peptides in visceral pain seems to be different and it is likely that opioids are not largely involved in modulation of visceral pain (183).

Therefore we conclude that based on the results from our study, the mechanisms involved in esophageal pain perception are not likely to be dependent on endogenous opioid release. Although we cannot exclude efficacy of exogenously administered opioid agonists in the control of esophageal hypersensitivity, our results suggest a less prominent role for the opioid neurotransmitter system in modulation of visceral pain. In addition, these findings are consistent with clinical impression that opioids are of limited use in the treatment of visceral pain in functional GI disorders.

5.3 Blocking the serotonin system

5.3.1 Introduction

Anxiety and depression were found to be associated with increased severity of GERD-related symptoms and reduced quality of life scores (89). In addition, within the GERD population, patients with a poor reflux-symptom correlation reportedly exhibit a higher level of anxiety compared to patients with a good reflux-symptom correlation (159, 190). Kahrilas *et al.* described the presence of psychogenic factors such as hyperalgesia, allodynia, hypervigilance, and increased anxiety, as an alternative explanation for PPI-refractory GERD symptoms (190). Extensive research has revealed that serotonin plays a pivotal role in the regulation of GI function and has long been associated with emotion regulation and psychological disorders such as depression, anxiety and phobia (191, 192). The exact role of serotonin or 5-hydroxytryptamine (5-HT) in the brain-gut axis is still incompletely understood, however serotonin can be considered as one of the key components of the brain-gut axis model. Serotonin is a major neurotransmitter predominantly found in the GI tract mainly in mucosal enterochromaffin cells and in the CNS.

Knowledge on the modulating role of serotonin in GI function and the brain-gut axis has mainly been obtained from the use of selective serotonin reuptake inhibitors (SSRIs) such as citalopram or other serotonin receptor agonists. Our group previously demonstrated that acute administration of citalopram significantly lowered chemical and mechanical esophageal sensitivity in hypersensitive HV (115). On the contrary, when a study was performed in normosensitive HV, no effect of citalopram on esophageal sensitivity was found. Similar observations were made when the effect of buspirone, a partial 5-HT_{1A} receptor agonist, on esophageal sensitivity to multimodal stimulation in HV was investigated (193). On the other hand, it was shown that buspirone is able to modify esophageal motility by enhancing the esophageal peristaltic amplitude in health (194). These data emphasize the fact that altered serotonin availability has no clear-cut effects on esophageal sensation and GI sensation in general. The presence of many types of 5-HT receptors in the GI tract and the lack of suitable and selective 5-HT receptor antagonists for use in human research could be a plausible explanation for these discrepancies. One possible method to overcome this problem is the application of the acute tryptophan depletion (ATD) technique.

ATD temporarily reduces the availability of the essential amino acid tryptophan (TRP), which is the precursor of serotonin and thereby ATD decreases the synthesis of serotonin. ATD is a validated technique to acutely lower central and peripheral serotonin concentrations (195). TRP depletion is accomplished by administration of an amino acid mixture lacking TRP. The ATD technique is widely used in psychiatric research to investigate the role of central serotonin in affective disorders. Further research also demonstrated that ATD affects GI physiology by delaying gastric emptying and enhancing visceral pain perception during rectal balloon distention (196, 197). Furthermore, ATD has been shown to alter gastric postprandial motor function and distention-induced nausea. These findings establish involvement of serotonin in the control of gastric accommodation and sensitivity (198).

The aim of the current study was to use the ATD method to investigate the effect of blocking the serotonin system on esophageal sensitivity in a group of HV. In this way, we hope to further unravel the role of serotonin in esophageal sensation in HV and thereby gain more insight in the involvement of the serotonin system in symptom perception in rGERD patients.

5.3.2 Materials and methods

5.3.2.1 Acute tryptophan depletion method

Three factors are involved in the synthesis of 5-HT: i) the concentration of free TRP in blood plasma, ii) the amount of free TRP that is able to cross the blood-brain-barrier (BBB), iii) the activity of the TRP hydroxylase enzyme which is the rate limiting step in 5-HT synthesis. 5-HT synthesis can be influenced by interfering with any or all of these factors. An acute drop in plasma levels of TRP can be achieved by administration of an amino acid load which does not contain TRP (195). First of all, administration of an amino acid mixture stimulates protein synthesis in the liver, which depletes plasma levels of free TRP and secondly, the amino acid mixture includes large neutral amino acids (LNAA) which compete with TRP for transport across the BBB, thereby reducing the entry of TRP in the brain (Figure 5.7).

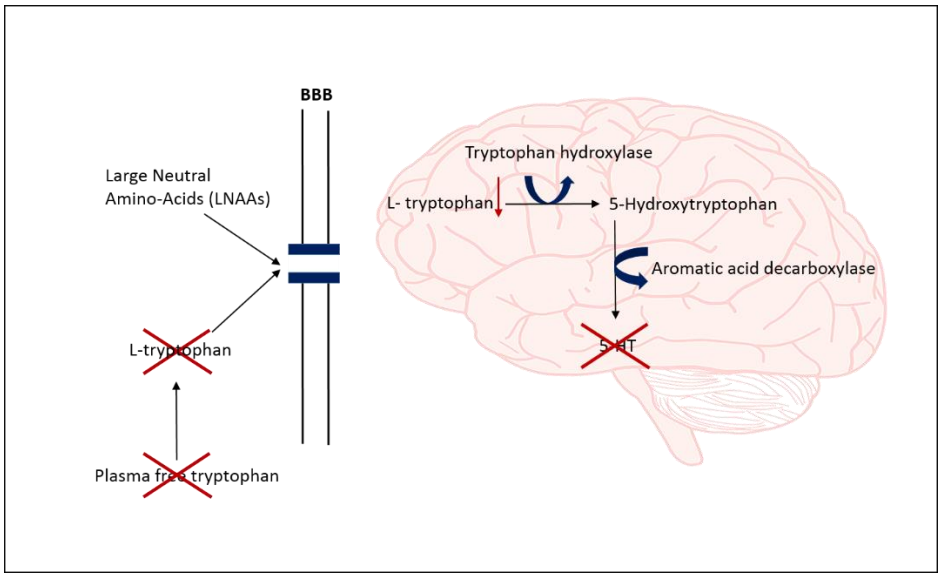

Figure 5.7 Acute tryptophan depletion method. The administration of an amino acid mixture stimulates protein synthesis in the liver and depletes plasma levels of free TRP. The mixture includes large neutral amino acids (LNAA) which compete with TRP for transport across the BBB, thereby reducing the entry of TRP in the brain and impairing the synthesis of 5-HT. Abbreviations: BBB=blood brain barrier, LNAAs=large neutral amino acids, 5-HT= 5-hydroxytryptamine or serotonin.

The amino acid mixture for the current study was prepared according to a protocol previously used by our group (198). All substrates were commercially available with an isotopic and chemical purity of minimal 99%. The identity of the products was confirmed using gas chromatography-mass spectrometry (GC-MS, GC-column: AT5-MS 30m x 0.25 mm internal diameter; 0.25µm film (Grace)). The mixture consisted of 15 amino acids including: 4.1g L-alanine, 2.4g glycine, 2.4g L-histidine, 6.0g L-isoleucine, 10.1g L-leucine, 6.7g L-lysine, 4.3g L-phenylalanine, 9.2g L-proline, 5.2g L-serine, 4.3g L-threonine, 5.2g L-tyrosine, 6.7g L-valine,

3.7g L-arginine, 2.0g L-cysteine, 3.0g L-methionine and 3.0g L-tryptophan. The TRP-deficient amino acid mixture consisted of the same 15 amino acids but was lacking TRP.

5.3.2.2 Study design

We performed this study in HV according to the outline shown in Figure 5.8. Since the amino acid mixture has an influence on the levels of brain 5-HT synthesis of the study participants, the Mini International Neuropsychiatric Interview (Dutch, version 5.0.0, DSM-IV) was used at the recruitment of the volunteers to evaluate their psychosocial condition. Based on the outcome of the neuropsychiatric interview, candidates were considered eligible for participation in the study.

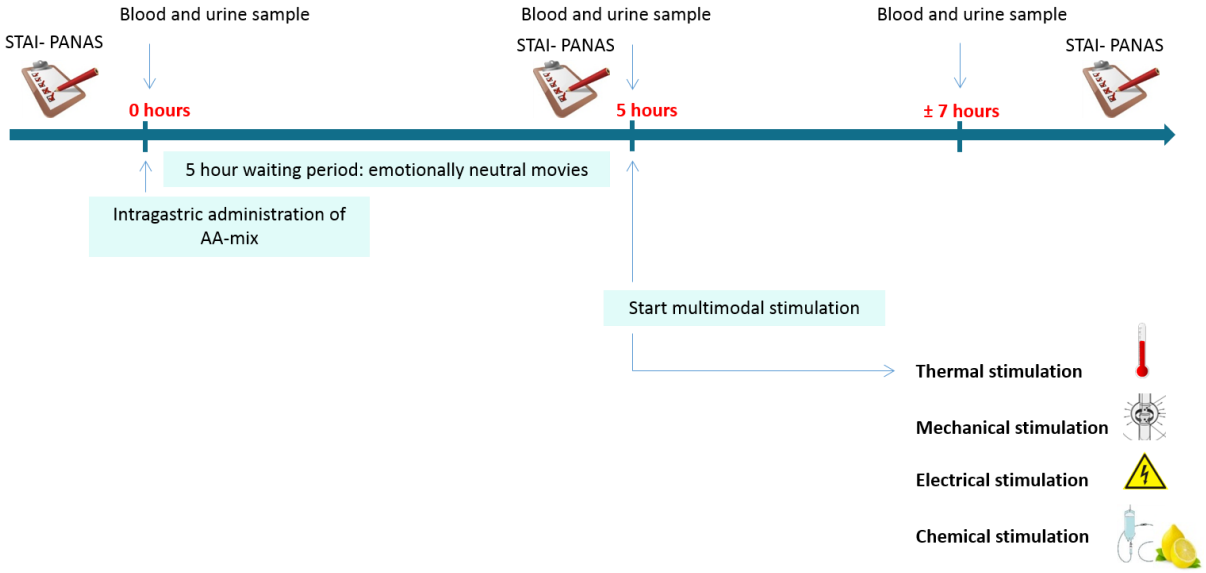

Figure 5.8 Outline of the acute tryptophan depletion study. Abbreviations: STAI=State-Trait Anxiety Inventory, PANAS=Positive and Negative Affect Schedule, AA-mix=amino acid mixture.

Esophageal sensitivity was evaluated during the multimodal esophageal stimulation protocol at two different study days, after administration of an amino acid mixture containing TRP (control) and after an amino acid mix without tryptophan (ATD), with an interval of at least one week. Sessions were run in a single-blind way. The order of the control condition and ATD condition was randomized using a randomization tool (www.randomization.com).

After an overnight fast, the amino acid mixture (control mix or ATD mix) was administered directly into the stomach via a nasogastric catheter (RT12/100, polyurethane enteral feeding tube, Eurosteriel Medical, Dronten, NL) to avoid nausea due to the unpleasant taste and smell of the mixture. Since maximal TRP depletion is obtained approximately 5 hours after intake of the amino acid mixture, the mixture was administered through nasogastric infusion 5 hours

prior to the actual start of the multimodal esophageal stimulation test. Blood samples were collected at baseline (T=0), T=5h and T=7h to measure plasma TRP levels, plasma ratio TRP/∑LNAA (sum of tyrosine, leucine, phenylalanine, isoleucine, valine). Furthermore, urine samples were collected to measure levels of urinary 5-hydroxyindoleacetic acid (5-HIAA) which is the most important metabolite of 5-HT. The analysis of these biochemical parameters were performed by the Laboratory Medicine unit of the University Hospital (Leuven, Belgium).

During the time between administration of the amino acid mixture and the actual start of the multimodal esophageal stimulation test, study participants were asked to watch standardized movies with a neutral emotional content. Five hours after the administration of the amino acid mixture, the multimodal stimulation probe was positioned in the distal esophagus. Emotional status of the study subjects was assessed using the STAI-state and PANAS questionnaires at time point T0, T5 and T7.

5.3.2.3 Statistical analysis

The number of participants to be included was calculated with a medium comparison test (comparison of variance, GPower 3.1.9.2 software). In order to detect a 10% difference with a risk α of 5%, we needed to include 15 volunteers in total to ensure a power of 90%. Thermal, mechanical, electrical and chemical sensitivity was measured at 1st perception, PPT and PTT and these thresholds were used to assess esophageal sensitivity. Statistical analysis was performed using GraphPad Prism 7.02 (GraphPad Software, Inc., La Jolla, CA USA). Esophageal sensitivity for the four different stimulation modalities was compared between ATD and control conditions using two-tailed paired *t*-test or Wilcoxon matched pairs test depending on the distribution of the data which was evaluated using the Kolmogorov-Smirnov test. Two-way ANOVA with a post-hoc *t*-test per time point with Bonferroni correction for multiple testing was used to evaluate the change in parameters of interest over time in male and female volunteers. A p-value of 0.05 was considered to be statistically significant. P-values were corrected for multiple testing using Bonferroni correction. Data are presented as median [25th-75th percentile], unless stated otherwise.

5.3.2.4 Ethical approval

The protocol was approved by the ethics committee of the University Hospital of Leuven (approval number: S57087) and registered at ClinicalTrials.gov (NCT03017768). Written informed consent was obtained from participants before inclusion in the study.

5.3.3 Results

Fifteen HV were included in this study protocol (7m/8f, mean age 24 years [21y-33y]). Seven out of 8 female volunteers experienced nausea during the ATD condition. In comparison, in the condition with the placebo amino acid mixture, 4 out of 8 female HV reported nausea. The occurrence of side effects was not different between the ATD and placebo condition (p= 0.28, Fisher's exact test). Two out of 7 male volunteers reported nausea in the ATD condition, 1 out of 7 male HV reported nausea in the placebo condition. No difference in the occurrence of nausea was present between the 2 conditions in male HV (p>0.9999, Fisher's exact test). Women reported significantly more nausea than men (p=0.04, Fisher's exact test).

The biochemical parameters are shown in Table 5.1: baseline values (T0) are comparable under both conditions. ATD significantly reduced plasma levels of TRP 5 hours and 7 hours after administration of the amino acid mixture (p<0.0001). Calculation of the ratio of TRP and the sum of LNAAs (TRP/∑LNAA) revealed the same convincing drop in plasma TRP levels. In urine samples, the levels of 5-HIAA, which is the major metabolite of 5-HT, were significantly decreased at T5 and T7 after ATD compared to the control condition (Table 5.1).

Table 5.1 Biochemical parameters at time point 0, time point 5 and time point 7 during the ATD condition and control condition.

	T0	T5	T7
TRP (µmol/L)			
Control	65.0 [49.3-69.5]	141.9 [102.6-168.3]***	73.6 [59.4-105.0]***
ATD	62.5 [51.8-74.8]	7.4 [5.0-18.2]	10.5 [6.8-15.6]
TRP/∑LNAA (x100)			
Control	12.2 [11.2-16.0]	10.2 [9.0-11.1]***	8.5 [7.3-11.4]***
ATD	12.7 [10.4-15.2]	0.5 [0.3-1.2]	0.9 [0.6-2.3]
5-HIAA (mg/L)			
Control	3.9 [1.9-5.7]	2.4 [1.4-4.1]**	1.4 [0.9-1.0]**
ATD	3.5 [1.9-4.5]	1.0 [0.5-2.0]	0.7 [0.0-1.3]

Results are presented as median [25th –75th percentile], n=15. **p<0.01, *** p<0.0001, between-group differences. All significant p-values survive Bonferroni correction. ATD=acute tryptophan depletion, TRP=tryptophan, LNAA= large neutral amino acids, 5-HIAA= 5-hydroxyindoleacetic acid.

When comparing ATD to the control condition, we found no influence on esophageal sensitivity to thermal stimulation. The thresholds for pain perception and pain tolerance were not altered after administration of the amino acid mixture lacking TRP (PPT p=0.19, PTT p=0.08) (Figure 5.9 A,B). Similar results were found for mechanical (PPT: p=0.71, PTT: p=0.05) and electrical stimulation (1st perception: p=0.50, PPT: p=0.39): ATD did not alter the sensitivity thresholds compared to the control mixture (Figure 5.9 C-F).

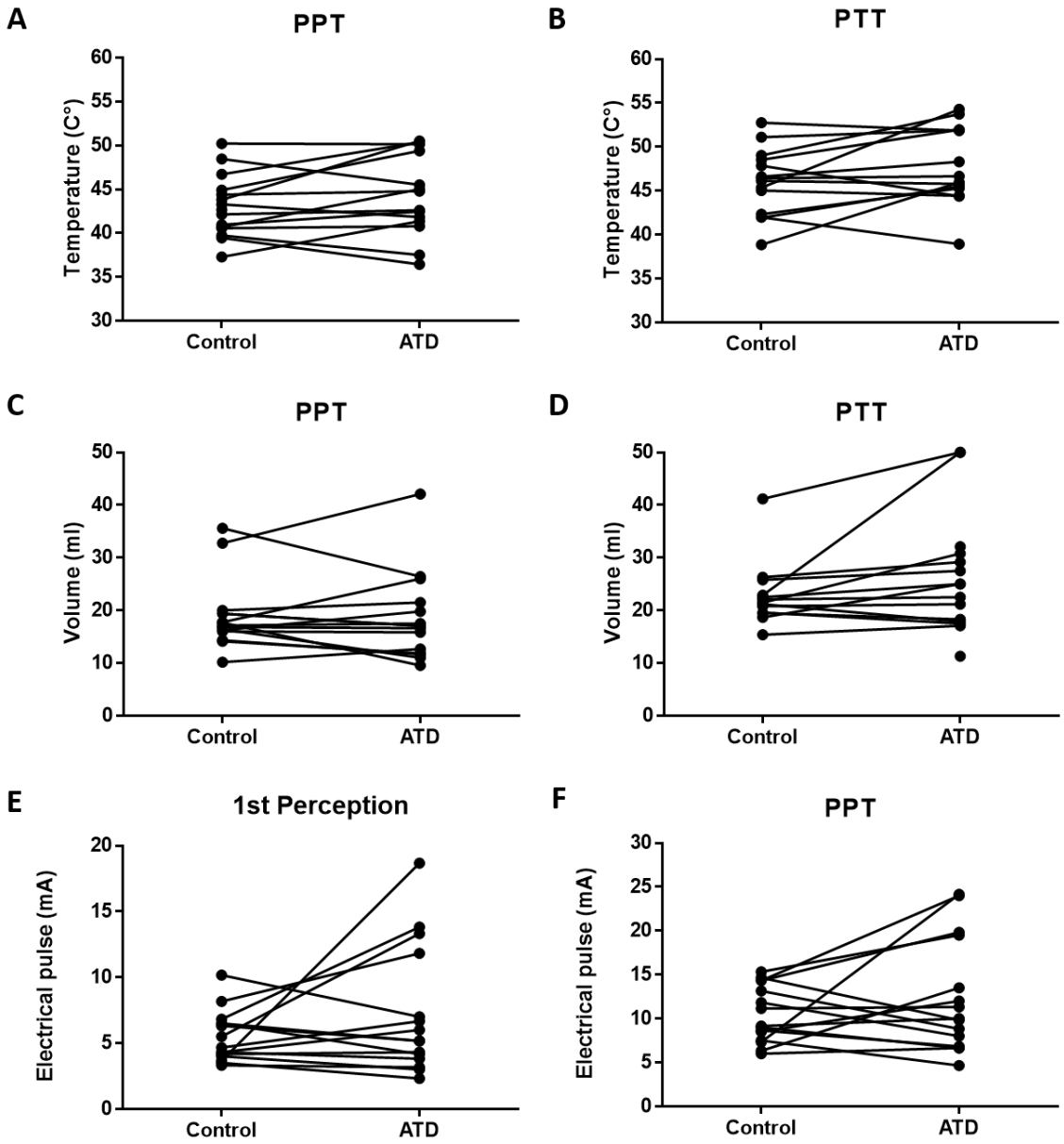

Figure 5.9 Results of esophageal multimodal stimulation after ATD or the control condition. No alterations in esophageal sensitivity to **A,B)** thermal, **C,D)** mechanical and **E,F)** electrical stimulation were seen when the two study conditions were compared. Abbreviations: ATD= acute tryptophan depletion, PPT= pain perception threshold, PTT=pain tolerance threshold.

ATD did decrease PPT during chemical stimulation (p=0.0172) with a pronounced effect size (Cohen's d^{+}=0.67) (Figure 5.10B). No effect on the other two sensitivity thresholds (1st perception: p=0.21, PTT: p=0.36) was found (Figure 5.10 A,C). In comparison with the previous study no ceiling effect was observed, all HV reached the sensitivity thresholds for chemical stimulation.

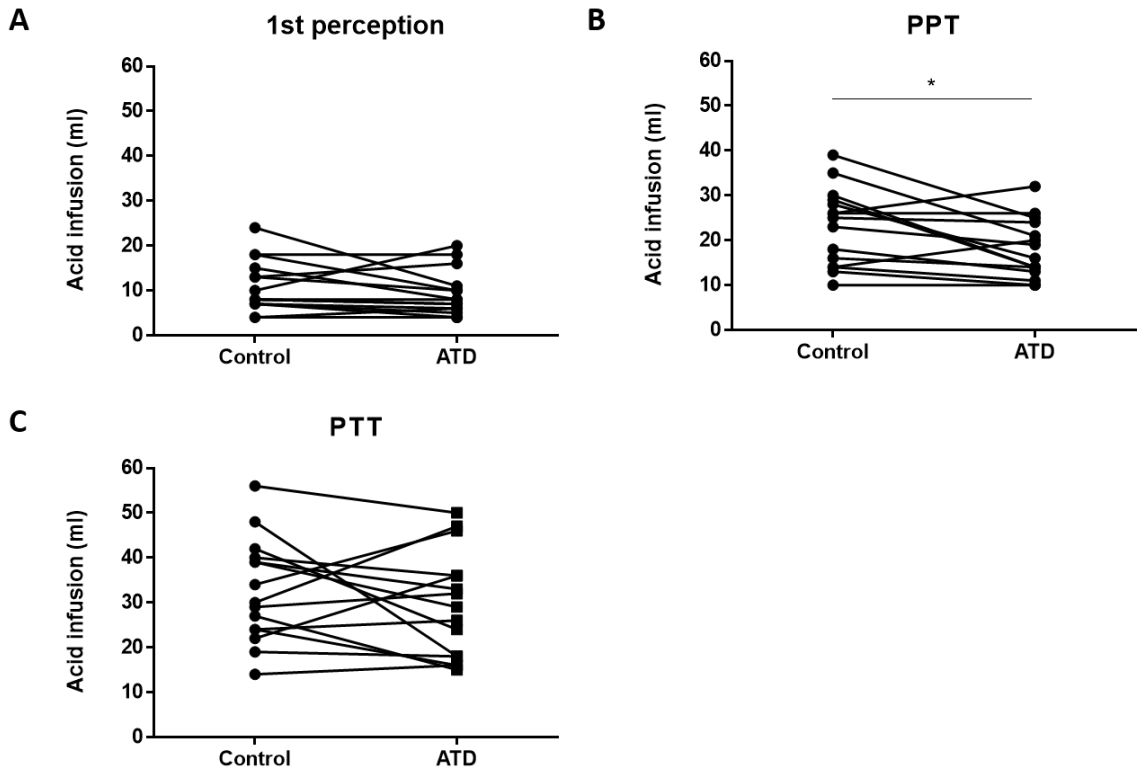

Figure 5.10 Results of esophageal chemical stimulation after ATD or in the control condition. **A,C)**No differences were seen for the 1st perception threshold and PTT. **B)** A significant decrease in PPT was seen after ATD compared to control. * $p<0.05$, corrected for multiple testing. Abbreviations: ATD=acute tryptophan depletion. PPT=pain perception threshold, PTT=pain tolerance threshold.

When we further looked in to the differences in PPT between placebo and ATD, we found that there was a gender difference: women appeared to be more sensitive to acid infusion compared to men in both conditions ($p=0.002$). However, this difference was not more pronounced by ATD. Women did not respond significantly stronger to ATD than men, there was no interaction effect of gender and treatment ($p=0.96$) (Figure 5.11).

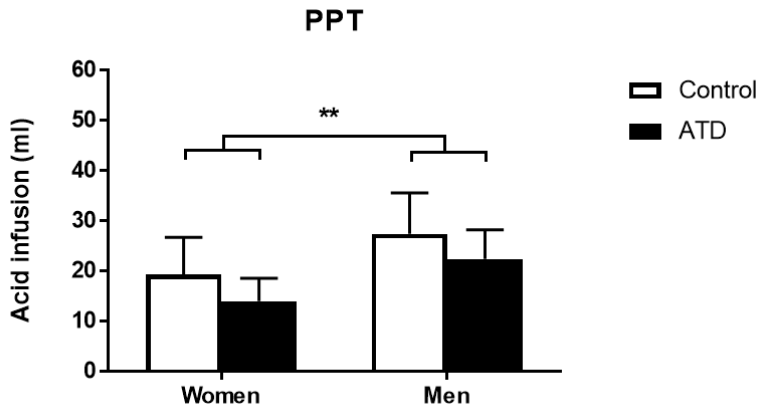

Figure 5.11 Comparison of chemical stimulation between women and men. Two-way ANOVA revealed a significant difference between the volume of acid infusion at which women reached PPT compared to the PPT threshold in men. There was no interaction effect of ATD and gender. **p<0.01, corrected for multiple testing. Abbreviations: ATD=acute tryptophan depletion, PPT=pain perception threshold.

As mentioned above, there was no effect of ATD on esophageal sensitivity to thermal, mechanical and electrical stimulation. However, we performed a two-way ANOVA analysis to investigate the effect of gender and as was the case with the chemical stimulation, gender differences were also present for thermal and mechanical sensitivity. For thermal stimulation, thresholds for PPT and PTT (p=0.0058 and p=0.0001, respectively) were lower in women than in men. Thresholds for mechanical stimulation were significantly lower in women than in men (PPT: p=0.008, PTT: p=0.03). No gender differences were seen for electrical stimulation (1st perception: p=0.24, PPT: p=0.53).

No differences in positive and negative affect scores were present at T0, T5 and T7 in ATD or control condition (Figure 5.12 A,B). Also STAI-State scores remained stable throughout the study period in the ATD as well as in the control condition (Figure 5.12C).

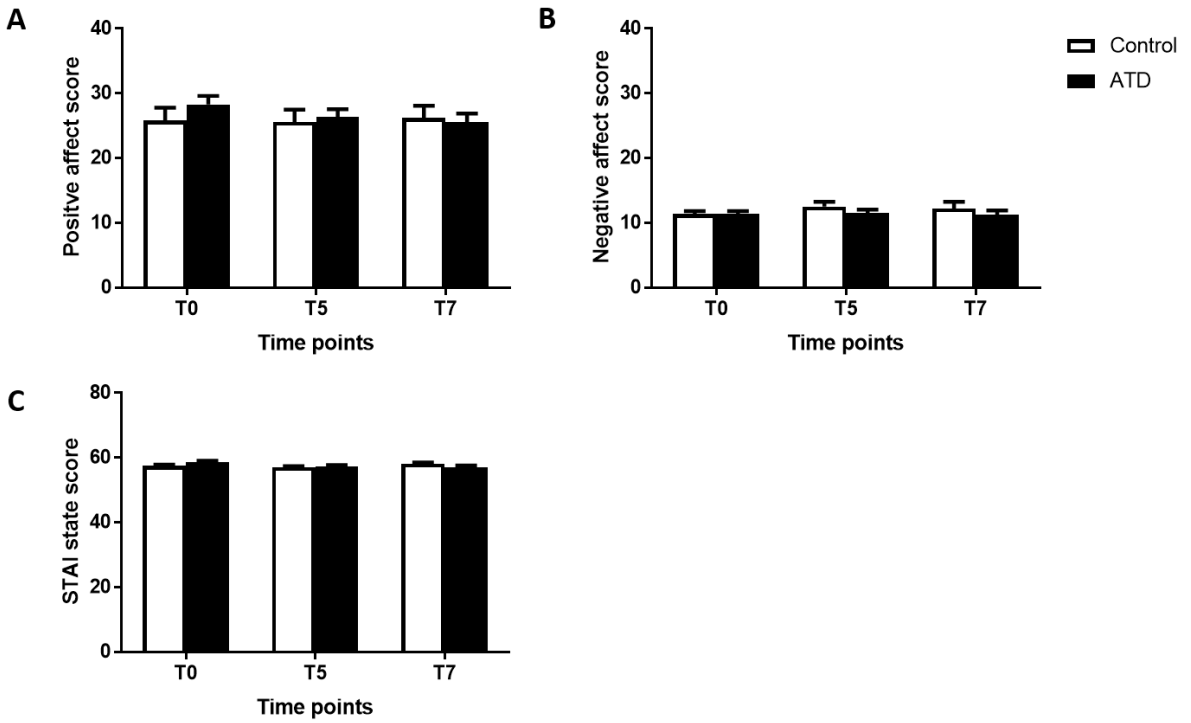

Figure 5.12 Results of **A)** Positive affect and **B)** Negative affect scores and **C)** STAI-state questionnaire scores before and after multimodal stimulation in the control and ATD condition. No significant differences in questionnaire scores were observed. Abbreviations: STAI=State-Trait Anxiety Inventory, PANAS=Positive and Negative Affect Schedule.

5.3.4 Discussion

The exact role of the serotonin system in the modulation of GI function, the involvement in visceral sensitivity and functional GI disorders is not fully elucidated yet. Studies on the influence of 5-HT on GI function are mainly performed using serotonin agonists such as SSRIs, while the effect of serotonin antagonism is studied less extensively. Therefore, the aim of this experiment was to evaluate the effect of low levels of peripheral and central 5-HT, achieved by acute tryptophan depletion, on esophageal sensitivity to multimodal stimulation in healthy volunteers. ATD is an established technique using the ingestion of an amino acid load that lacks tryptophan, the precursor of serotonin, to deplete the levels of this essential amino acid. It has been established that reducing the plasma levels of TRP causes a consequent reduction in 5-HT synthesis (195, 199).

The biochemical analysis of blood and urine samples at 3 different time points in our study protocol confirmed that plasma levels of TRP decreased in all subjects as a result of ATD. The ratio of plasma TRP/∑LNAAs, which is considered to be an accurate predictor of brain TRP levels (200), was significantly lower after ATD compared to the ingestion of the control mixture. The concentration of 5-HIAA, the most important metabolite of 5-HT synthesis, was also significantly lower after ATD compared to the control condition. Based on the results of the biochemical analysis we concluded that ATD was effective in all of our study participants.

The major finding of this study was an increase in esophageal sensitivity to acid infusion when levels of TRP were depleted. Furthermore, we observed a differential effect of multimodal esophageal stimulation in men and women. Women had lower thresholds for pain perception and pain tolerance to temperature, mechanical and chemical stimulation in comparison with men. ATD did not enhance this gender difference. Finally, no effect of ATD on anxiety scores and positive and negative affect scores was present.

Previous studies of our group investigated the effect of the 5-HT_{1A} agonist buspirone and citalopram, a SSRI, on esophageal sensitivity to multimodal stimulation. No alteration in esophageal sensitivity to multimodal stimulation could be demonstrated in HV. In contrast to this previous experiments, where 5-HT agonists were used, we investigated the influence of blocking the serotonin system by ATD. We found a significantly lower pain perception threshold during esophageal chemical stimulation in HV. The differences in study outcome can be explained by the differential effects of 5-HT agonists or blocking the serotonin

neurotransmitter system. Furthermore, in contrast to receptor agonists, ATD alters the general availability of peripheral and central serotonin and does not act in a receptor specific fashion. The fact that ATD lowers sensitivity thresholds to acid infusion, can be an indication that normal levels of 5-HT are important in mediating esophageal (acid) sensitivity. Alterations in 5-HT regulation are associated with comorbidities such as anxiety and depression (89). Therefore, our findings may have implications for the understanding and treatment of patients with rGERD or functional heartburn since in this population psychosocial comorbidities such as anxiety, are known to be more frequent (16, 89).

In this study we observed a differential effect of esophageal multimodal stimulation in women and men. When we compared the sensitivity thresholds for women and men in the control condition and after ATD, we found that for temperature, mechanical and chemical stimulation, women were more sensitive for all thresholds compared to men. This is partially in accordance with findings of a study by Krarup *et al.* The authors found that women had lower pain thresholds to mechanical stimulation of the esophagus and also a smaller number of women tolerated the maximum acid challenge during chemical stimulation. In contrast to our results, there were no differences between men and women for thermal stimulation. Similar to our experiment also no differences were present between men and women for electrical stimulation (201). Nguygen *et al.* reported a lower pain threshold to balloon distention in women compared to men (202). In a study by Reddy *et al.* the opposite results were reported: men appeared to be more sensitive to esophageal balloon distention than women. However, the authors of the latter study conclude that not balloon volume or pressure are valid to score sensory responses but rather strain is associated with stimulation of mechanosensitive receptors. Based on measurements of strain, no differences were found between mechanical sensitivity in men and women (203). In this study, it was also demonstrated that women have larger referred pain areas than men, indicating a differential mechanism of central pain processing (203). The authors postulate that men are more able to inhibit visceral pain at the central level and conclude that this may contribute to the observation of a female predominance in functional GI disorders since an aberrant central processing of pain signals is one of the hypotheses explaining functional disorders (63, 120, 169, 203).

ATD did not have a measureable effect on mood in our study participant. This finding is in agreement with other studies in which anxiety ratings have been recorded following ATD. When healthy volunteers were subjected to ATD, very little effects on anxiety scores were reported (199, 204, 205). Although we did not find an alteration in mood or anxiety scores in HV, the effects of ATD on mood and anxiety are dependent on the characteristics of the study population: mood alterations after ATD have been described in patients with a history of depression, and changes in anxiety scores were present in patients with social anxiety disorders (199).

The exact mechanism by which ATD influences esophageal sensitivity is not fully clear: ATD works on 5-HT on central and peripheral levels. Apart from measurements of 5-HIAA in urine samples, we did not assess the peripheral level of esophageal 5-HT concentration, so we are not able to make a definite conclusion if the sensitivity changes to acid infusion are mediated by actions of peripheral or central 5-HT availability. Since the ratio of TRP/∑LNAAs was decreased massively after ATD, we hypothesize that it is more likely that the mechanisms of ATD induced alterations in acid sensitivity are centrally mediated.

A possible limitation of this study was the fact that the majority of our female healthy volunteers had side effects after the administration of the amino acid mixture. Women experienced mild nausea during both the control condition and the ATD condition, although feelings were more pronounced during ATD condition (reported by the subjects during the study visits and observations of the study investigators). These feelings of nausea were only present in female volunteers and therefore could be a potential explanation why women react more sensitive to esophageal stimulation than man. The fact that we only observed gender differences in this study and not in the stimulation tests where opioid antagonists and chlorpromazine were used, further confirms this hypothesis.

In conclusion, ATD altered sensitivity to acid perfusion: the pain perception threshold was significantly lower compared to the condition where a control mixture was used. ATD did not affect the 1st perception threshold or PTT to chemical stimulation. Also the other stimulation modalities were unaffected by ATD. It remains to be further investigated whether ATD alters local GI 5-HT concentrations. More research is needed to clarify the exact role of 5-HT in esophageal sensitivity and acid sensitivity in particular. From our study results, we conclude that the apparent involvement of 5-HT in acid sensitivity underline the utility of so called

'neuromodulators' in the treatment strategy of patients with reflux hypersensitivity and function heartburn.

5.4 Blocking the dopamine system

5.4.1 Introduction

The study of the interaction between psychological state and GI function is complex. It is known that anxiety is one of the factors that have an influence on visceral sensitivity in humans (67, 159). Dopamine, a predominant catecholamine neurotransmitter in the mammalian brain, controls a wide range of differential functions including emotion (*e.g.* anxiety), cognition, positive reinforcement and locomotor activity. Besides its numerous actions in the brain, dopamine is known to play an important role in a variety of functions in the periphery such as regulation of food intake and GI function (206-208).

The dopaminergic system has been the focus of a lot of research over the past 30 years, mainly because several pathological conditions such as Parkinson's disease, schizophrenia, and Tourette's syndrome, have been linked to a dysregulation of dopaminergic transmission. In addition, it has been postulated that the dopamine system is also involved in the regulation of motility of the upper GI tract. Expression of dopamine receptors in human LES has been demonstrated by several studies. In the early nineties, Missale *et al.* suggested the presence of both the D_1 and D_2 receptor in the LES (209). Furthermore, Liu and colleagues confirmed the presence of D_1 and D_2 in the LES and demonstrated that both of these two receptors are present in the circular muscle of distal esophageal body. Based on their results, the authors postulated an important role for dopamine in esophageal function (210).

The available literature indicates that dopamine decreases LES pressure and inhibits gastroduodenal motility (210-212). Indeed, in the 1970s and 1980s, D_2 receptor antagonists *e.g.* metoclopramide and domperidone became available and have shown to counteract these effects: dopamine antagonists elicit an increase in LES pressure and stimulate gastroduodenal motility and gastric emptying (211, 213). They have consequently proven to be of value in certain cases of gastroparesis, and in relieving nausea and vomiting. Based on the findings of several smaller studies, dopamine antagonists also appear to be useful in the management of reflux esophagitis (16, 213). Itopride which is a combined D_2 receptor antagonist and an acetylcholinesterase inhibitor was used in healthy subjects and was shown to reduce the occurrence of TLESRs and reflux events (214). In a one-month, uncontrolled trial of 26 patients with GERD, 100mg of itopride decreased esophageal acid exposure and improved reflux symptoms (215).

The effect of dopamine antagonists on visceral hypersensitivity has been less explored. The effect of chlorpromazine on visceral hypersensitivity in a rat model for irritable bowel syndrome (IBS) has been investigated and the authors conclude that chlorpromazine seems to have a beneficial effect on visceral hypersensitivity to colorectal distention (216). Chlorpromazine, which is a D_2 receptor antagonist, has been used in clinical practice as one of the first anti-psychotic drugs since 1952 (217). Various disorders such as schizophrenia and autism in adults and children, short term treatment of anxiety, severe hiccups, nausea, vomiting and severe pain are treated by the use of chlorpromazine (218, 219).

The aim of this third study was to investigate the involvement of the dopaminergic neurotransmitter system in regulation of esophageal sensitivity. Since there are indications that chlorpromazine has effects on visceral sensitivity in rats, a first step in this project was to investigate the effect of dopamine antagonism by IV administration of chlorpromazine, on esophageal sensitivity in a group of HV.

5.4.2 Materials and Methods

5.4.2.1 Study design

Two separate sessions were scheduled for each subject: a placebo and a chlorpromazine condition, with at least one week interval. Sessions were ran in a double-blind, randomized-controlled way. The order of placebo and chlorpromazine administration was randomized (www.randomization.com) and this scheme was carried out by an experienced independent researcher who also prepared the chlorpromazine or placebo solution for infusion.

Due to the potent sedative effect of chlorpromazine, the recommended administration dose was an IV infusion of 10 mg (217). Chlorpromazine is rapidly absorbed and widely distributed in the body: plasma half-life is approximately 30 hours and the elimination of metabolites may be prolonged. Whilst plasma concentration of chlorpromazine itself rapidly declines, excretion of chlorpromazine metabolites is very slow (217). Ten mg chlorpromazine for IV infusion was added to 100mL NaCl 0.9%, infusion ran over 30 minutes.

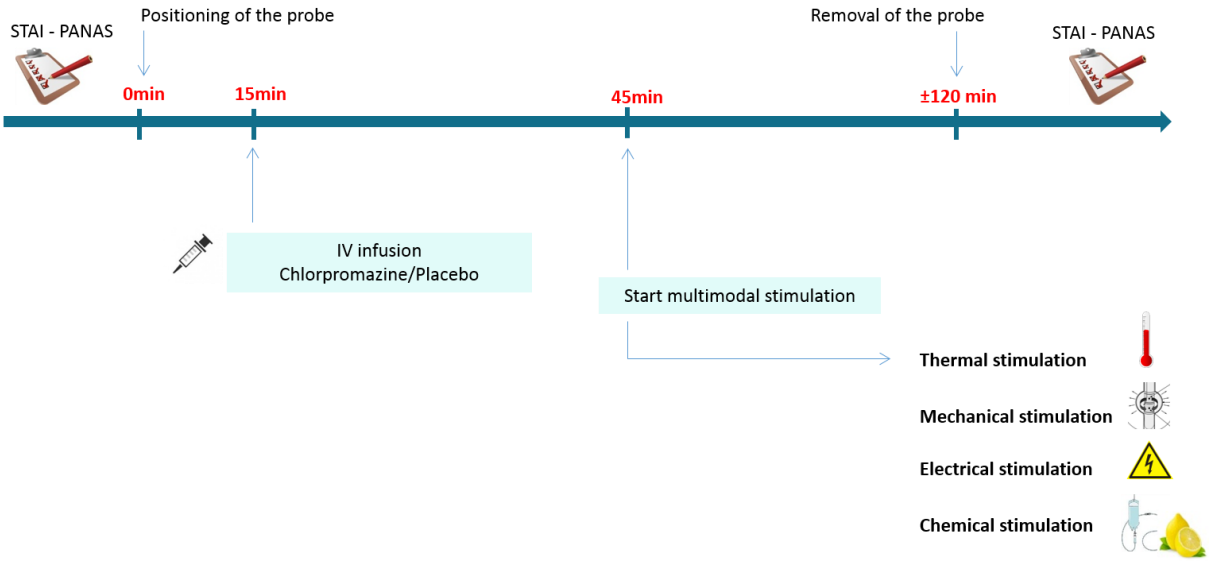

Figure 5.13 Outline of the chlorpromazine study. Abbreviations: STAI=State-Trait Anxiety Inventory, PANAS=Positive and Negative Affect Schedule, IV=intravenous.

After an overnight fast, subjects were asked to fill out the STAI-state and PANAS questionnaires to assess their emotional status before the onset of the stimulation tests (131).

After placement of the multimodal esophageal stimulation probe, blood pressure was monitored and each subject was submitted to an electrocardiogram (ECG) since prolongation of the QT interval is a possible side effect of chlorpromazine. Hereafter, 10mg of chlorpromazine or placebo (saline) dissolved in 100mL of saline, was slowly administered over

a time period of at least 30 minutes via IV infusion. During placebo sessions, 2mL saline (NaCl 0.9%) was administered to 100mL of saline. When infusion was completed, the multimodal stimulation test was initiated (Figure 5.13).

5.4.2.2 Statistical analysis

The number of volunteers to be included was calculated with a medium comparison test (comparison of variance). Using GPower 3.1.9.2 software we specified the following parameters: α error probability of 5%, effect size of 0.87 and a power of 85%. Based on these parameters we needed to include 14 volunteers in order to highlight the expected differences. Our main objective was to show a difference for multimodal stimulation with or without administration of chlorpromazine in all of the randomized healthy subjects. The individual perception thresholds of the participants during multimodal stimulation were used to determine changes in esophageal sensitivity. Statistical analysis was performed using GraphPad Prism 7.02 (GraphPad Software, Inc., La Jolla, CA USA). Esophageal sensitivity for the four different stimuli was compared between chlorpromazine and placebo condition using two-tailed paired *t*-tests or Wilcoxon matched-pairs signed-rank test in case of nonparametric data distribution, evaluated by the Kolmogorov-Smirnov test. Fisher's exact tests were used to evaluate if there was a difference in the occurrence of a ceiling effect for the sensitivity thresholds during the stimulation tests. A p-value of 0.05 was considered to be statistically significant. P-values were corrected for multiple testing using Bonferroni correction. Data are presented as median [25^{th}-75^{th} percentile], unless stated otherwise.

5.4.2.3 Ethical approval

The protocol was approved by the ethics committee of the University Hospital of Leuven (approval number: S60403) and the Federal Agency for medicines and health products (EudraCT number: 2016-003131-38). Furthermore, the study was registered at ClinicalTrials.gov (NCT03183310). Written informed consent was obtained from participants before inclusion in the study.

5.4.3 Results

Thirteen HV (7m/6f, mean age 25 years [range 19-40]) were enrolled in this study. All participants experienced a mild sedative effect of IV administration of chlorpromazine. Besides drowsiness and the feeling of being tired, no other side effects were reported by the HV during the course of the experiment. In the placebo condition, no side effects were observed in any of the participants.

When comparing the two study conditions we found no influence of chlorpromazine on esophageal sensitivity to thermal stimulation. When IV administration of 10mg chlorpromazine was compared to placebo, we found no significant changes in the thresholds for PPT (45.57°C [41.34-49.31] vs. 45.96°C [43.81-47.57], p=0.64) and PTT (48.87°C [45.26-51.83] vs. 49.36°C [47.52-50.12], p=0.47) (Figure 5.14 A,B).

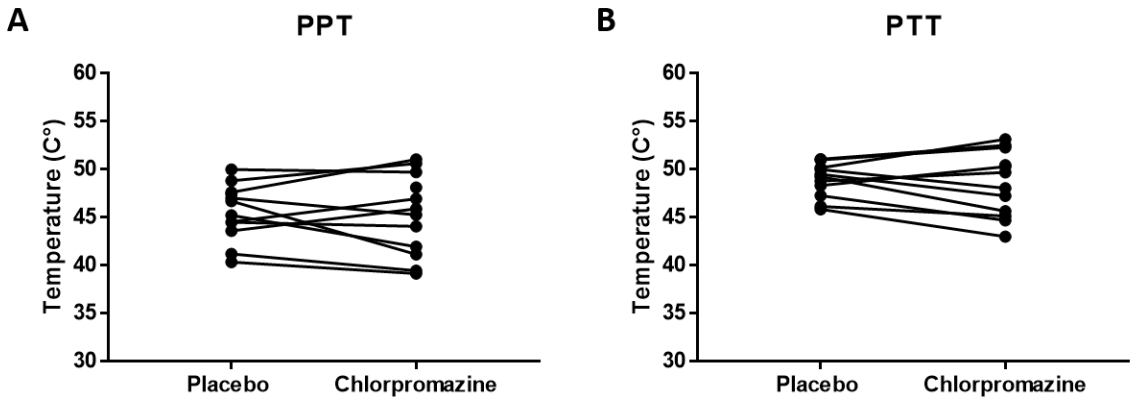

Figure 5.14 Results of esophageal temperature stimulation after IV administration of chlorpromazine or placebo. No alterations in **A)** PPT and **B)** PTT to temperature stimulation were seen when the two study conditions were compared. Abbreviations: PPT= pain perception threshold, PTT=pain tolerance threshold

Similar results were found for esophageal mechanical stimulation (Figure 5.15 A,B). The volume at which HV reached PPT and PTT was not altered after administration of chlorpromazine compared to placebo (PPT: 19.15mL [17.9-25.65] vs. 24.33mL [21.80-28.35], p=0.53, PTT: 24.80mL [14.20-32.60] vs. 28.95mL [24.65-32.83], p=0.51). Similar to the stimulation test with naloxone and methylnaltrexone, we observed a minor ceiling effect: a small proportion of the participants (2 out of 13 for PPT, 2 out of 13 for PTT) did not reach the sensitivity thresholds at 50mL during the two study visits and were not included in the analysis of mechanical sensitivity. The number of participants that reached the sensitivity thresholds during balloon distention was similar in both study conditions (PPT: p=0.48, PTT: p=0.22).

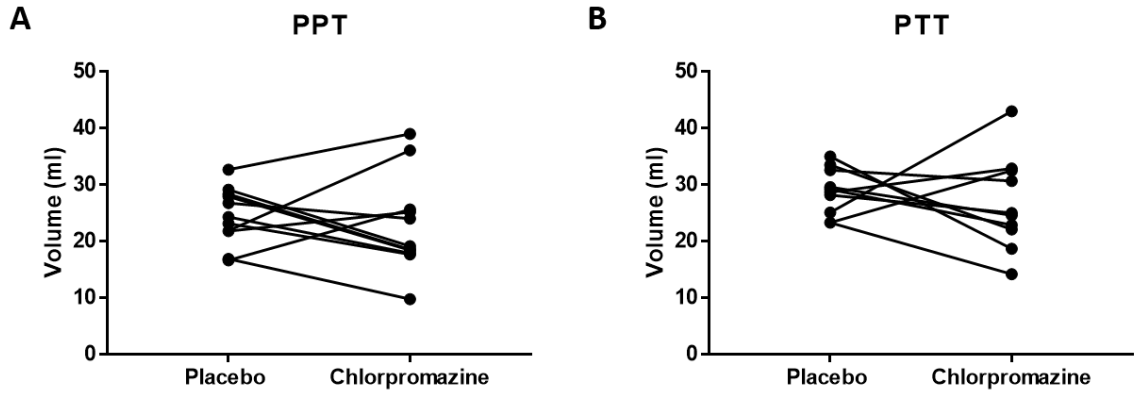

Figure 5.15 Results of esophageal mechanical stimulation after IV administration of chlorpromazine or placebo. No alterations in **A)** PPT and **B)** PTT to mechanical stimulation were seen when the two study conditions were compared. Abbreviations: PPT= pain perception threshold, PTT=pain tolerance threshold.

When comparing esophageal sensitivity to electrical stimulation after administration of chlorpromazine to the placebo condition, no differences were seen for the 1st perception threshold (5.67mA [4.42-11.33] vs. 7.83mA [5.34-9.75], p=0.75), nor for PPT (11.83mA [9.00-19.25] vs. 13.17mA [9.75-18.67], p=0.81) (Figure 5.16 A,B).

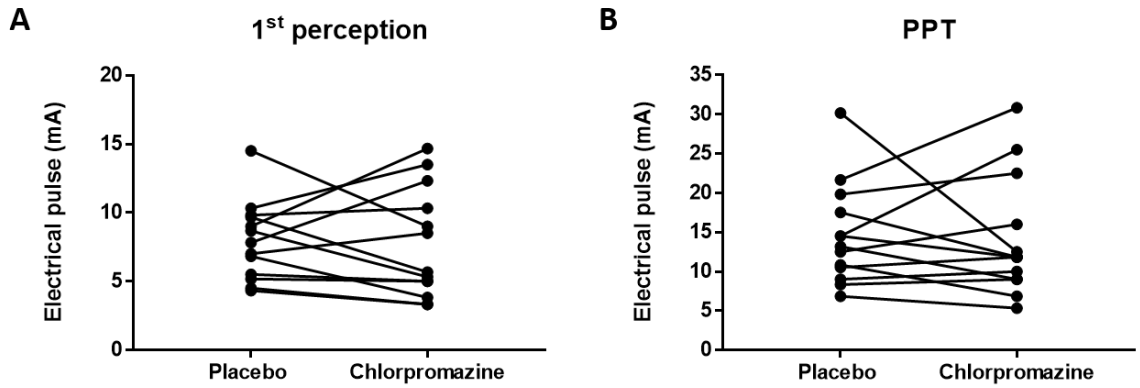

Figure 5.16 Results of esophageal electrical stimulation after IV administration of chlorpromazine or placebo. No alterations in **A)** 1st perception threshold and **B)** PPT to electrical stimulation were seen when the two study conditions were compared. Abbreviations: PPT= pain perception threshold.

As shown in Figure 5.17 sensitivity thresholds for chemical stimulation of the esophagus by infusion of an acid solution did not change after chlorpromazine administration compared to placebo (1st perception: 12.50mL [6.13-24.50] vs. 10.00mL [4.50-18.00], p=0.18, PPT: 13.00mL [10.00-28.00] vs. 19.00mL [12.75-34.00], p=0.39, PTT: 28.50mL [15.75-39.50] vs. 29.00mL [17.50-42.50], p=0.84). Similar to the mechanical stimulation, we also observed a ceiling effect during the chemical stimulation. Three out of 13 HV for PPT and 4 out 13 for PTT, did not reach the sensitivity thresholds after 30 minutes of acid infusion in both study visits and were not included in the analysis. The number of participants that reached the sensitivity thresholds

during acid infusion was similar in both study conditions (PPT: p=0.48, PTT: p=0.46, Fisher's exact test).

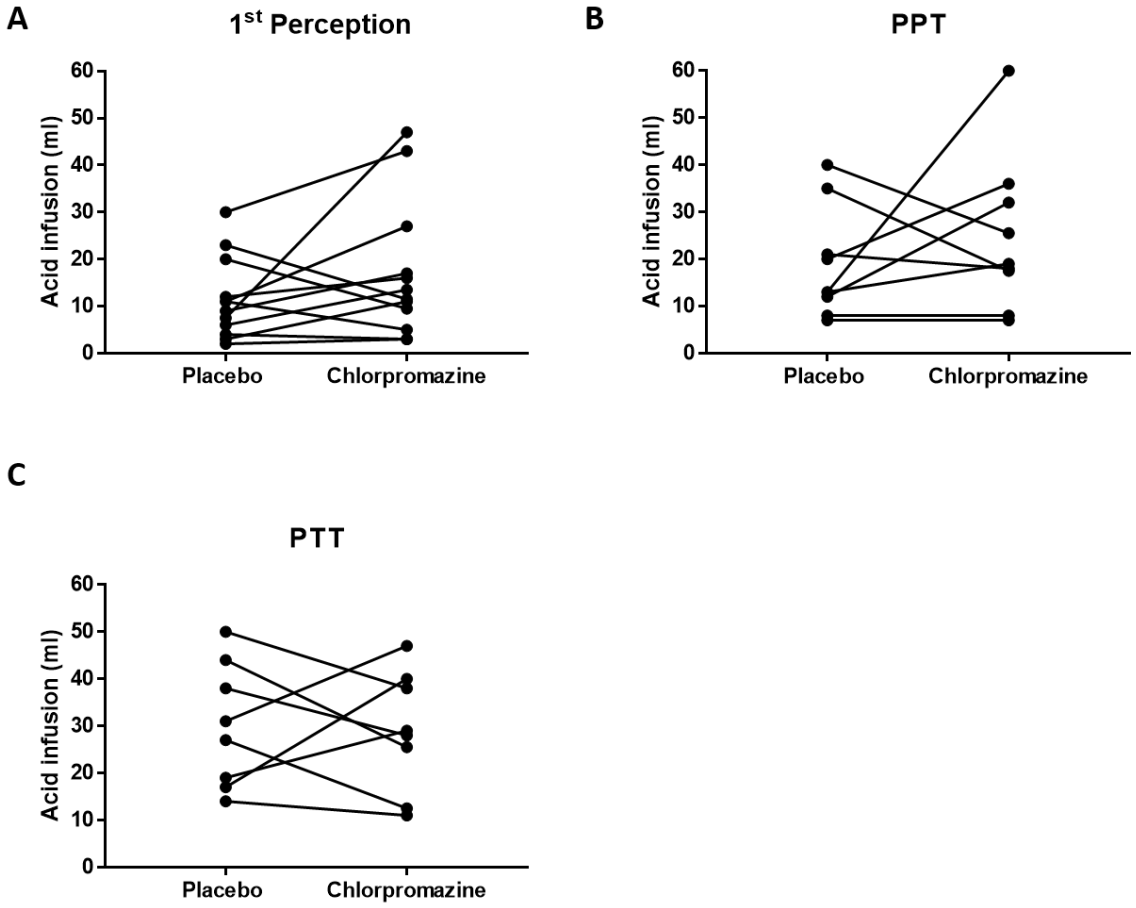

Figure 5.17 Results of esophageal chemical stimulation after IV administration of chlorpromazine or the placebo condition. No differences in acid sensitivity were present when chlorpromazine was compared to the placebo condition. Threshold for **A)** the 1st perception threshold, **B)** PPT and **C)** PTT remained unchanged. Abbreviations: PPT=pain perception threshold, PTT=pain tolerance threshold.

We performed a two-way repeated measures ANOVA analysis to investigate the effect of gender on the results of the four different stimulation modalities. For thermal stimulation, thresholds for PPT and PTT were not different in women in comparison with men (p=0.79 and p=0.97, respectively). Similarly, thresholds for mechanical stimulation were similar in women and in men (PPT: p=0.85, PTT p=0.30). Furthermore, no gender differences were seen for electrical stimulation (1st perception: p=0.34, PPT: p=0.14) and chemical stimulation (1st perception: p=0.12, PPT: p=0.26, PTT: p=0.65).

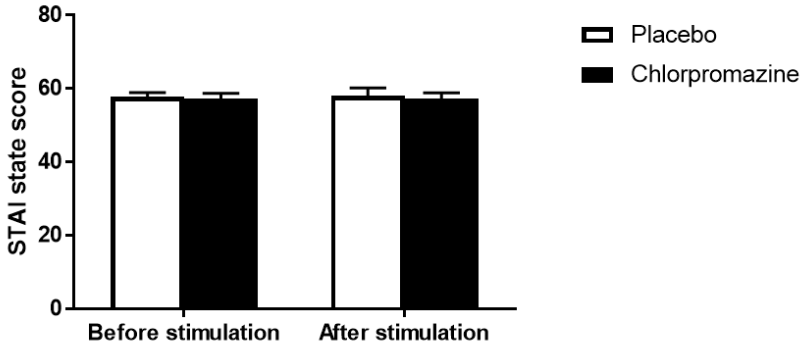

Figure 5.18 STAI scores before and after the multimodal esophageal stimulation for the placebo and chlorpromazine condition. Scores were similar at the end of the study protocol compared to the STAI scores before the start of the stimulation in both conditions.

STAI scores were similar in both conditions (Figure 5.18). No differences in STAI scores were present between placebo and chlorpromazine after the stimulation test (58.00 [56.25-59.75] vs. 57.00 [56.25-58.00], p=0.12).

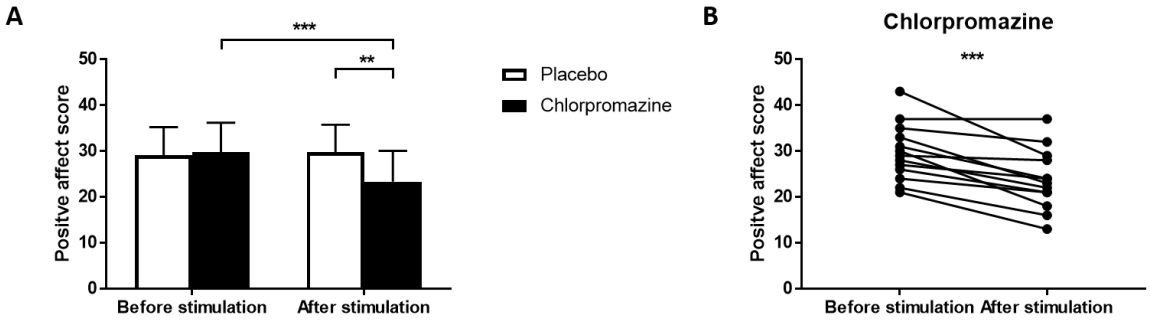

Figure 5.19 Positive affect scores in the placebo condition and after administration of chlorpromazine, affect scores were assessed before and after the stimulation test. **A, B)** Significant lower positive affect scores were present after the multimodal stimulation in the chlorpromazine condition compared to positive affect scores before the start of the stimulation. These positive affect scores were also significantly lower in the chlorpromazine condition compared to the placebo condition. ** p<0.01, *** p<0.001.

Using the PANAS questionnaire, positive affect scores were assessed before and after the stimulation test in the placebo condition and after administration of chlorpromazine (Figure 5.19A). Significantly lower positive affect scores were present after the multimodal stimulation in the chlorpromazine condition compared to positive affect scores before the start of the stimulation (23.00 [19.50-28.50] vs. 29.00 [25.00-34.00], p=0.0002) (Figure 5.19B). Positive affect was also significantly lower in the chlorpromazine condition compared to the placebo condition (23.00 [19.50-28.50] vs. 28.00 [25.00-33.00], p=0.004). There was a significant interaction effect of treatment (p=0.0016).

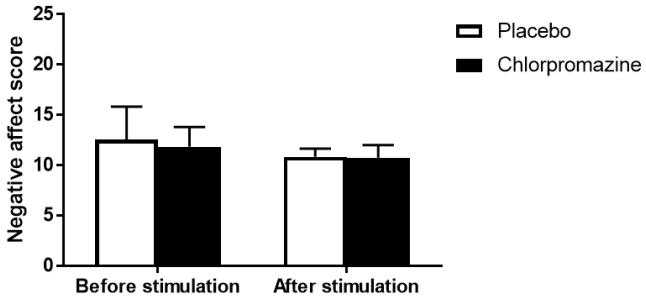

Figure 5.20 Negative affect score in the placebo condition and after administration of chlorpromazine, affect scores were assessed before and after the stimulation test. Numerically lower negative affect scores were present after the multimodal stimulation in the placebo condition compared to negative affect scores before the start of the stimulation.

Additionally, also negative affect scores were assessed before and after the stimulation test (Figure 5.20). A trend towards lower negative affect scores was present after the multimodal stimulation in the placebo condition compared to negative affect scores before the start of the stimulation (11.00 [10.00-11.50] vs. 12.00 [10-.00-14.00], p=0.05) (Figure 5.20). However, significance was lost after Bonferroni correction.

5.4.4 Discussion

The influence of different anti-nociceptive pathways in esophageal sensitivity needs further research. Therefore as a third part of this project we investigated the role of the dopamine system in esophageal sensation. We chose to use chlorpromazine which is a typical anti-psychotic drug with D_2 antagonistic properties. Chlorpromazine is traditionally used for regulation of positive and negative symptoms and cognitive symptoms in the treatment of schizophrenia. Furthermore it is used in the treatment of nausea, vomiting and severe hiccups (216, 220).

In the current study we compared esophageal sensitivity to multimodal stimulation in HV after the IV administration of 10mg of chlorpromazine and placebo. Our main finding was that chlorpromazine did not have an influence on esophageal sensitivity to thermal, mechanical, electrical and chemical stimulation. No changes in sensitivity thresholds to multimodal stimulation were observed. Assessment of emotional status before and after the stimulation using STAI-state and PANAS questionnaires revealed that HV had lower positive affect scores after the stimulation test when chlorpromazine was administered. Negative affect scores were lower after the stimulation test in the placebo condition, no changes in negative affect were observed in HV after administration of chlorpromazine.

Studies evaluating the influence of dopamine on esophageal function have mainly focused on its effect on motility. Several studies indicate that dopamine decreases LES pressure and gastric motility, therefore the use of dopamine antagonists such as itopride has been proposed for the treatment of esophageal motility disorders and GERD (214, 215) and in patients with functional dyspepsia (221). Data on the involvement of dopamine in visceral sensitivity are limited. One study investigated the effect of chlorpromazine on visceral hypersensitivity in a rat model for IBS. Observations in this study indicate that chlorpromazine has a beneficial influence on visceral hypersensitivity, however the inhibitory effect of chlorpromazine on visceral hypersensitivity is likely to be mediated by inhibition of 5-HT_{2A} receptor and not by the antagonistic properties of chlorpromazine on the D_2 receptor (216).

The influence of chlorpromazine on positive and negative affect scores is not surprising since this drug has long been used as anti-psychotic treatment in disorders such as schizophrenia and other psychiatric conditions such as bipolar disorder, where the effects of chlorpromazine are mainly beneficial for counteracting the symptoms of psychoses (222).

A limitation of the study is the lack of specificity of the agent, due to antagonistic activity of chlorpromazine at various receptors besides the dopamine D_2 receptor, including 5-HT_{2A} receptors and histamine receptors (223). Therefore, we could not exclude the possibility that other effects of chlorpromazine, including histamine H_1 receptor antagonism and anti-calmodulin activity, may also be involved in observations made in this study (223).

Another limitation of this study was the fact that IV administration of 10mg of chlorpromazine had a sedative effect in all our study participants. Although the administered dose was lower than doses used in clinical practice, a sedative effect was observed in all participants. This compromises the interpretation of our study results since we cannot exclude that a potential effect of chlorpromazine administration on esophageal sensitivity was masked by the sedation that was present. However, we have chosen to use chlorpromazine since it is a well-known drug with predictable side effects. Another major advantage of chlorpromazine is the fact that we could administer it intravenously, in this way plasma levels were better controlled than *e.g.* with drugs that need to be administered orally (first-pass metabolism).

In conclusion, we found no effect of chlorpromazine on esophageal multimodal stimulation in HV indicating that antagonism of the D_2 receptor is not likely to be involved in the modulation of esophageal pain perception. Other anti-nociceptive pathways besides the dopamine system are probably more important in the regulation of esophageal sensation.

CHAPTER 6

ALTERATIONS IN ESOPHAGEAL PAIN PERCEPTION DUE TO STRESS

The data represented in this chapter have been published in a slightly different form in the following publication:

Broers C, Melchior C, Van Oudenhove L, Vanuytsel T, Van Houtte B, Scheerens C, Rommel N, Tack J, Pauwels A. The effect of intravenous corticotropin-releasing hormone administration on esophageal sensitivity and motility in health. *Am J Physiol Gastrointest Liver Physiol.* 2017 May 1;312(5):G526-G534.

6 Alterations in esophageal pain perception due to stress

6.1 Introduction

The underlying mechanism of refractory typical reflux symptoms remains largely unclear. Numerous factors, including absence of underlying GERD, inadequate intake of PPIs, ongoing weakly acid reflux and esophageal hypersensitivity have been implicated (224, 225). Amongst these, a key role has been attributed to esophageal hypersensitivity, which is demonstrable in a large subset of patients with PPI-refractory GERD symptoms (128, 225).

The mechanisms underlying esophageal hypersensitivity have not been fully elucidated, however stress is considered a potentially important underlying factor (126, 127). Up to 64% of individuals with heartburn report that psychological factors including life stress, aggravate their GERD-related symptoms (147). Fass and colleagues showed that auditory stress exacerbated symptom perception during esophageal acid perfusion in GERD patients (87).

However, stress is not only able to alter esophageal sensitivity, but may also affect esophageal motility. As early as 1962, Rubin *et al.* showed a significant increase in non-peristaltic contractions during a stressful condition in healthy volunteers (226). More recent studies have also documented esophageal motility changes in response to stressors in healthy subjects and patients with pre-existing esophageal dysmotility abnormalities (227-229).

Stress induces the release of peripheral corticotropin releasing hormone (CRH) which is a pivotal player in the stress response of the GI tract. CRH plays a key role in the acute regulation of stress and anxiety-related behaviors and in the regulation of endocrine responses during chronic stress via activation of the hypothalamic pituitary adrenal axis (HPA-axis) (121, 122). During acute and chronic stress, CRH drives secretion of adrenocorticotropic hormone from the pituitary, ultimately leading to the release of cortisol from the adrenal glands (121). The effect of stress on the GI tract is at least in part mediated via a direct effect of CRH on the CRH_1-receptor identified on human intestinal mucosal mast cells and to lesser extent via CRH_2-receptors (230).

In this study, CRH was adminstered to mediate one of the key molecules involved in the GI stress response. We hypothesized that CRH will increase multimodal esophageal sensitivity and alter esophageal motility. Hence, we investigated whether administration of CRH affects esophageal sensitivity to thermal, mechanical, electrical and chemical stimulation in healthy volunteers (HV) and whether the CRH is involved in alterations in esophageal motility.

6.2 Materials and methods

6.2.1 Study population

Both sensitivity and motility studies were performed in HV. Prior to the initiation of the study, all participants provided informed consent. Both study protocols have been registered to ClinicalTrials.gov (NCT02736734, NCT02674256) and the European Union Drug Regulating Authorities Clinical Trials (EudraCT) registry under the numbers 2014-000602-36 and 2014-002239-33 and were in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Leuven (approval numbers S56177 and S57111).

6.2.2 Test conditions

After an overnight fast, HV came to the endoscopy unit of the university hospital. All study visits started between 1pm and 3pm to minimize diurnal variation. CRH administration was executed as follows: a solution of 100µg CRH powder for injection (CRH ferring®, Ferring, Aalst, Belgium) in 1 mL of NaCl 0.9% was injected IV over the course of 1 minute (231). This dose of CRH is known to alter GI function and increases plasma adrenocorticotropic hormone secretion to stress levels with detectable plasma CRH in humans (232, 233). Furthermore, this dose has been previously shown to reproduce the GI effects of stress through a mast-cell-dependent fashion (231). Side-effects were limited to transient facial flushing lasting from 5 to 45 minutes in approximately 75% of subjects. Intravenous CRH administration is clinically used as a diagnostic tool in locating the source of hypercortisolaemia in Cushing's disease. Following IV administration of 100µg CRH, maximal plasma concentrations of CRH are achieved after 5 minutes. The elimination half-life of one dose 100µg CRH is approximately 9 minutes. Cortisol levels reach a maximal concentration approximately 30 minutes after CRH administration, cortisol normalizes 120 minutes after CRH administration (231).

In the first protocol, we investigated the effect of CRH on esophageal sensitivity using a multimodal stimulation protocol in which all participants underwent two conditions: (i) placebo (NaCl 0.9%) and (ii) CRH administration. Over time, each participant received placebo or CRH (cross-over, counterbalanced) with an interval at least of one week, in a single-blinded fashion.

In the second study protocol, esophageal motility was assessed before and after the administration of CRH on the same day by a standard high resolution impedance manometry (HRiM).

6.2.3 Esophageal sensitivity testing by multimodal stimulation

Esophageal sensitivity was evaluated by the multimodal esophageal stimulation protocol as described in Chapter 3, paragraph 3.3. The multimodal stimulation probe was positioned through the mouth in the distal esophagus. The subjects remained in a semi-recumbent position for the entire study period. After a 15 minute adaptation period, subjects received an intravenous injection of placebo (NaCl 0.9%) or CRH. Immediately after the injection, the stimulation test was performed according to our experimental design (Figure 6.1). Thresholds for first perception, pain perception threshold (PPT) and pain tolerance threshold (PTT) were recorded.

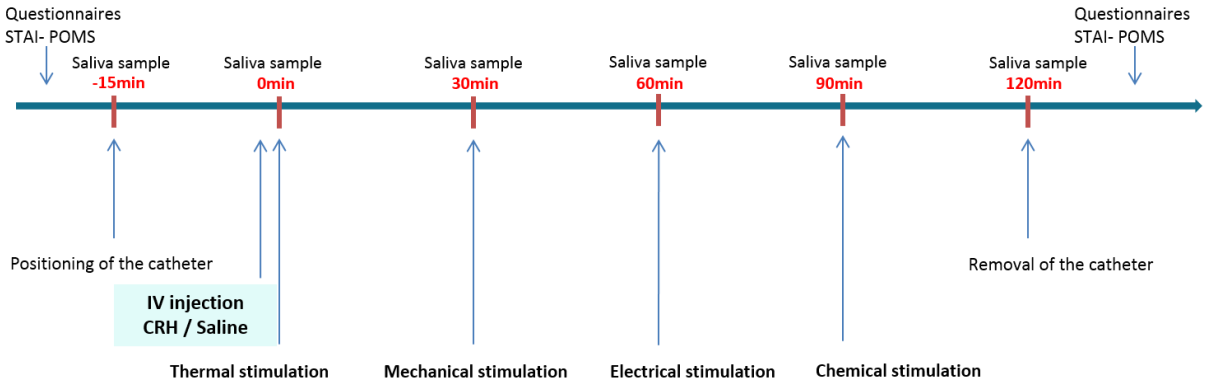

Figure 6.1 CRH-sensitivity study outline. STAI=state-trait anxiety inventory, POMS=profile of mood states, CRH=corticotropin-releasing hormone, IV=intravenous.

6.2.4 Esophageal motility testing by standard high resolution impedance manometry

Esophageal motility was assessed by standard HRiM as described in Chapter 3, paragraph 3.5. The HRiM catheter was placed transnasally and positioned along the esophagus with the distal 2 sensors in the stomach. After positioning of the catheter, subjects remained in a semi-recumbent position for the entire study period and pressure and impedance measurements were recorded.

In this protocol, test boluses of 5 mL liquid (water), 5 mL semi-solid (apple sauce) and 2cm^2 solid (white bread) were administered orally. All bolus stock contained 1% NaCl to enhance conductivity. Ten swallows of each consistency were executed. After measuring under baseline conditions, CRH was administered intravenously and after 30 minutes the same procedure was repeated (Figure 6.2).

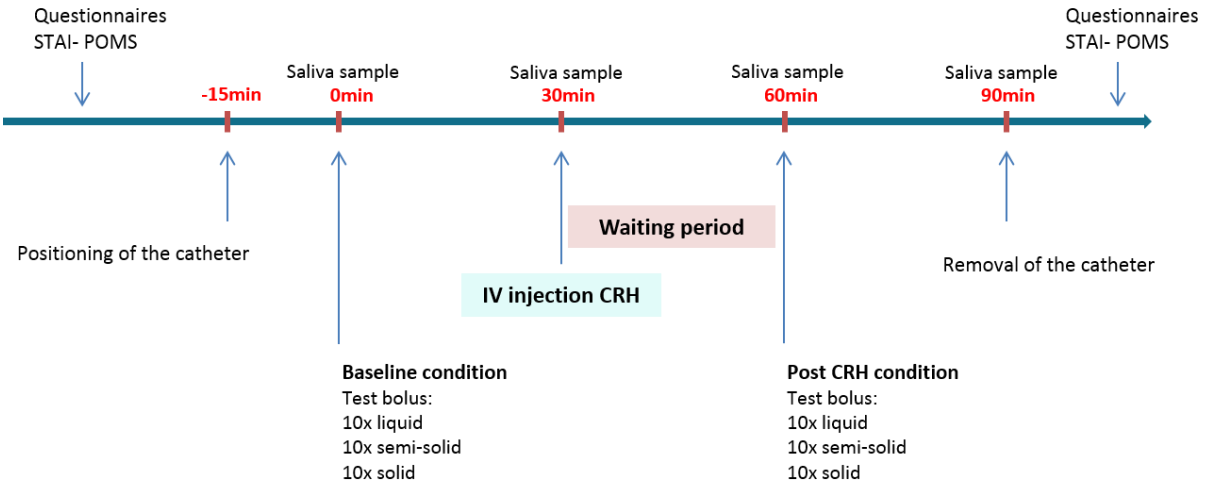

Figure 6.2 CRH-motility study outline. STAI=state-trait anxiety inventory, POMS=profile of mood states, CRH=corticotropin-releasing hormone, IV=intravenous.

Contraction patterns during swallows with different bolus consistencies were compared between baseline and CRH recordings according to Chicago Classification v3.0 (135). Esophageal contractile function was evaluated pre and post CRH by assessing the distal contractile integral (DCI), measuring contractile vigor and the intrabolus pressure (IBP). Furthermore, the integrated relaxation pressure of the LES, mean of the 4 seconds of maximal deglutitive relaxation in the 10 second-window beginning at upper esophageal sphincter (UES) relaxation, the IRP4 was calculated.

Furthermore, pressure flow analysis was performed using esophageal automated impedance manometry software (AIMPlot_OES_V4.2, copyright T. Omari, 2014). The following parameters were evaluated: (i) the ratio of nadir impedance to impedance at the time of peak pressure (NI/IIPP ratio or the impedance ratio, IR), which is used as a marker of bolus clearance, (ii) the intrabolus pressure slope (IBP slope), the rate of change in IBP recorded during the phase of transition from a full lumen to an occluded lumen. IBP slope is a marker of the pressurization needed to propel a bolus forward and (iii) pressure flow index (PFI) which reflects the relationship between IBP and bolus flow timing in the esophagus. The PFI is calculated using the formula (IBPxIBP slope)/(time from nadir impedance to peak pressure) and serves as global measure of pressure flow or EGJ resistance to bolus flow (137, 138).

6.2.5 Evaluation of stress symptoms and hormones, emotion and general mood

In both protocols, an assessment of momentary anxiety levels and mood state was performed by using the State-Trait Anxiety Inventory (STAI, state scale) and the Profile of Mood States (POMS) questionnaires before and after the study procedures.

Salivary samples were collected (Salivette, Sarstedt, Nümbrecht, Germany) to determine the concentration of salivary cortisol. In the sensitivity study, samples were collected before the positioning of the probe, immediately before the placebo or CRH administration, and every 30 minutes for 2 hours after administration of placebo or CRH (Figure 6.1). In the motility study, saliva samples were collected before the positioning of the probe, immediately before the CRH administration, and at 30 and 60 minutes after administration of CRH (Figure 6.2). The samples were stored at -20°C after centrifugation (4°C, 3000 rpm, 10 minutes). Salivary cortisol was determined by ELISA (DRG Diagnostics, Marburg, Germany) according to the manufacturer's instructions.

6.2.6 Statistical analysis

Statistical analysis was performed using Prism 5.01 (GraphPad Software, Inc, La Jolla, CA, USA). Threshold comparisons were performed as well as a comparison of differences in change in questionnaire data after and before the stimulations between CRH and placebo conditions within subjects. Comparisons were done using a paired Student's *t*-test or the non-parametric paired Wilcoxon signed rank test with Bonferroni correction for multiple testing. Deviations from Gaussian distribution were tested using the Shapiro-Wilk normality test. Cohen's d index, a measure for the size of observed effects, was performed for tests within groups using the mean and standard deviation. Cohen's d can be calculated as the difference between the means of two conditions divided by the pooled standard deviation (0.2=small effect, 0.5=medium effect, >0.8 large effect) (234). Results are expressed as median [25^{th} –75^{th} percentile] unless indicated otherwise. A p-value < 0.05 was considered statistically significant.

6.3 Results

6.3.1 Esophageal sensitivity

Fourteen HVs (8m/6f, 30.7±10.6 years, body mass index (BMI) 23.7±2.0 kg/m^2) were recruited to investigate the effect of CRH administration on esophageal sensitivity assessed by multimodal stimulation. Esophageal sensitivity to mechanical distention was significantly increased after CRH administration compared with placebo condition. After CRH administration, PPT levels during mechanical stimulation were reached at significantly lower distending balloon volumes compared to placebo administration (24.10ml vs. 28.48ml, p=0.0023, survives Bonferroni correction), with a large size effect (Cohen's d=0.89). Similarly, PTT levels were reached earlier after CRH than placebo administration (30.24 vs. 32.30ml, p=0.1953), with a small size effect (Cohen's d=0.42) (Table 1). However, this did not reach statistical significance since we only evaluated subjects reaching the PTT at the maximal inflation volume of 50mL. In addition, we observed that 6 (43%) HV did not reach PTT in the placebo condition at the maximal inflation volume, whereas this was only the case in 2 (14%) HV in the CRH condition (Fisher's Exact test, p=0.2087). Administration of CRH had no influence on esophageal sensitivity to thermal, electrical or chemical stimulation in HV compared to placebo condition (Table 6.1).

Table 6.1: Results of esophageal sensitivity tests.

	CRH	Placebo (Saline)	p-value uncorrected	Cohen's d +
Temperature stimulation (°C)				
PPT	43.99 [41.03-47.06]	45.13 [42.14-48.91]	0.27	0.22
PTT	46.48 [45.00-49.09]	49.07 [44.81-50.66]	0.35	0.19
Mechanical Stimulation (ml)				
PPT	24.10 [18.71-26.15]	28.48 [23.39-43.88]	**0.0023***	0.89
PTT (n=8)	30.24 [23.98-35.08]	32.30 [28.43-45.20]	0.20	0.42
Electrical stimulation (mA)				
1st perception	5.42 [4.45-9.58]	7.58 [5.00-10.00]	0.88	0.04
PPT	11.08 [8.0-16.38]	12.92 [9.38-15.38]	0.95	0.03
Chemical stimulation (ml)				
1st perception	12.00 [4.00-24.00]	12.00 [4.00-26.00]	0.55	0.08

Results are presented as median [25th –75th percentile], n=14. Correction for multiple testing was performed, *survives Bonferroni correction. + Effect size expressed as Cohen's d (0.2=small effect, 0.5=medium effect,>0.8 large effect). Mechanical stimulation: only volunteers reaching PTT at the maximal inflation volume (50ml) are included in the analysis. CRH=corticotropin-releasing hormone, PPT=pain perception threshold, PTT=pain tolerance threshold.

6.3.2 Esophageal motility

Fourteen HV (8m/6f, mean age 26.6±5.8 years, BMI 23.1±1.2 kg/m^2) were included in the study. After CRH administration, DCI values significantly increased for all three types of bolus (liquid p=0.0012, semi-solid p=0.0017, solid p=0.011, all survive Bonferroni correction), whereas no differences in IBP were seen. Finally, IRP values for all three bolus consistencies significantly increased after administration of CRH (liquid p=0.039, semi-solid p=0.0085, solid p=0.0039, except for liquid all survive Bonferroni correction) (Table 6.2).

Table 6.2: HRM results of esophageal motility tests.

	Pre CRH	**Post CRH**	**p-value** $_{uncorrected}$	**Cohen's d** +
Liquid				
DCI (mm Hg.s.cm)	686 [541.30-1149.00]	1391 [926.00-2035.00]	**0.0012***	0.94
IBP (mm Hg)	7.00 [5.00-8.25]	6.00 [4.50-8.00]	0.075	0.26
IRP (mm Hg)	8 [7-9]	12 [9-14]	**0.039**	0.62
Semi-solid				
DCI (mm Hg.s.cm)	620.50 [381.50-915.30]	1180.00 [639.80-1811.00]	**0.0017***	0.92
IBP (mm Hg)	5.00 [3.75-9.25]	5.00 [4.00-7.25]	0.79	0.02
IRP (mmHg)	8 [7-9]	10 [7-14]	**0.0085***	0.64
Solid				
DCI (mm Hg.s.cm)	1261.00 [832.80-2596.00]	1947.00 [1405.00-3329.00]	**0.0107***	0.63
IBP (mm Hg)	4.50 [2.75-8.50]	5.00 [2.75-8.50]	1.00	0.06
IRP (mm Hg)	8 [6-12]	12 [10-16]	**0.0039***	0.85

Changes in esophageal motility before and after IV CRH administration. Values for distal contractile integral (DCI), intrabolus pressure (IBP) and median integrated relaxation pressure (mIRP4) are shown for liquid, semi-solid and solid boluses. Results are presented as median [25th –75th percentile], n=14. Correction for multiple testing was performed for each bolus type. * survives Bonferroni correction. CRH=corticotropin-releasing hormone.

Differences in Chicago Classification v3.0 outcome before and after administration of CRH were assessed for all three bolus consistencies although Chicago Classification is currently only validated for liquid bolus swallows. No significant changes were seen when the Chicago Classification was applied to liquid or solid boluses. When the classification was applied to semi-solid boluses, a significant decrease in prevalence of ineffective esophageal motility (IEM) was found (pre CRH 6 out of 14 subjects, 42.86% compared to 0 out of 14, 0% after CRH, p=0.015).

6.3.3 Pressure flow analysis

The impedance ratio for liquid and semi-solid swallows decreased significantly after CRH administration (liquid p<0.0001, survives Bonferroni correction, semi-solid p=0.0327). No significant effect was reached for the difference in impedance ratio with solid boluses (p=0.059). Mean IBP slope (mmHg/s) increased after CRH administration for semi-solid and solid swallows (semi-solid p=0.0041, solid p=0.0003, all survive Bonferroni correction), no statistically significant increase was reached for liquid swallows (p=0.058). PFI increased for semi-solid (p=0.0017, survives Bonferroni correction) and solid swallows (p=0.0031, survives Bonferroni correction), no changes were seen for liquid swallows (p=0.1937) (Table 6.3).

Table 6.3: Pressure flow analysis metrics based on HRiM before and after IV CRH administration.

	Pre CRH	Post CRH	p-value $_{uncorrected}$	Cohen's d +
Liquid				
Impedance ratio	0.29 [0.22-0.34]	0.25 [0.20-0.28]	**<0.0001***	0.73
IBP slope (mm Hg/s)	2.12 [1.35-2.58]	2.57 [1.85-3.35]	0.06	0.46
PFI	5.47 [3.12-7.64]	6.20 [3.57-10.60]	0.19	0.31
Semi-solid				
Impedance ratio	0.36 [0.25-0.48]	0.29 [0.25-0.35]	0.03	0.63
IBP slope (mm Hg/s)	5.67 [3.72-7.65]	7.02 [5.57-8.86]	**0.0041***	0.65
PFI	32.25 [25.82-65.03]	51.50 [36.03-79.54]	**0.0017***	0.49
Solid				
Impedance ratio	0.47 [0.39-0.58]	0.43 [0.33-0.55]	0.06	0.25
IBP slope (mm Hg/s)	10.87 [5.10-15.42]	16.08 [12.09-21.81]	**0.0003***	0.96
PFI	140.80 [53.85-276.60]	223.00 [109.80-455.00]	**0.0031***	0.52

The ratio of mean nadir impedance and impedance at peak pressure (or impedance ratio), intrabolus pressure slope (IBP slope) and pressure flow index (PFI) are shown for liquid, semi-solid and solid boluses. Results are presented as median [25^{th} –75^{th} percentile], n=14. Correction for multiple testing was performed for each bolus type. * survives Bonferroni correction. CRH=corticotropin-releasing hormone.

Since the proximal esophagus is more sensitive to distention and proximal extent of reflux is an important factor for perception we additionally evaluated proximal esophageal function by pressure flow analysis. As shown in Table 6.4, CRH administration had no significant effect on any of the evaluated parameters in the proximal esophagus.

Table 6.4: Pressure flow analysis metrics of the proximal esophagus based on HRiM before and after IV CRH administration.

	Pre CRH	Post CRH	p-value $_{uncorrected}$
Liquid			
Impedance ratio	0.33 [0.32-0.35]	0.29 [0.26-0.32]	0.12
IBP slope (mm Hg/s)	9.87 [7.32-14.71]	14.00 [8.21-15.33]	0.32
PFI	17.35 [7.618-42.64]	30.23 [14.83-47.40]	0.30
Mean Peak Pressure (mm Hg)	50.03 [36.67-54.9]	48.15 [29.87-63.27]	0.83
IBP (mm Hg)	2.99 [1.47-4.89]	3.89 [2.33-4.69]	0.03
Semi-solid			
Impedance ratio	0.29 [0.26-0.35]	0.27 [0.24-0.31]	0.05
IBP slope (mm Hg/s)	16.15 [8.1521.45]	13.39 [10.75-17.26]	0.24
PFI	64.71 [39.33-147.00]	72.01 [44.03-110.4]	0.76
Mean Peak Pressure (mm Hg)	57.97 [42.42-67.1]	59.29 [45.23-65.08]	0.79
IBP (mm Hg)	5.61 [4.87-8.77]	6.96 [5.47-9.49]	0.21
Solid			
Impedance ratio	0.36 [0.31-0.45]	0.36 [0.30-0.40]	0.50
IBP slope (mm Hg/s)	25.24 [15.76-35.05]	21.29 [14.88-29.40]	0.11
PFI	216.2 [134.9-501.9]	146.9 [125.2-281.4]	0.17
Mean Peak Pressure (mm Hg)	78.45 [67.84-92.59]	76.44 [63.79-95.08]	0.76
IBP (mm Hg)	14.52 [10.36-16.55]	12.87 [10.52-17.74]	0.76

The ratio of mean nadir impedance and impedance at peak pressure (or impedance ratio), intrabolus pressure slope (IBP slope) and pressure flow index (PFI) are shown for liquid, semi-solid and solid boluses. Results are presented as median [25^{th} –75^{th} percentile], n=14. Correction for multiple testing was performed for each bolus type. CRH=corticotropin-releasing hormone.

6.3.4 Salivary cortisol, stress and mood

In the sensitivity study, salivary cortisol levels were compared at each time point between placebo and CRH conditions. CRH administration resulted in elevated salivary cortisol levels between 30 minutes and 120 minutes compared to placebo (Figure 6.3A). Cortisol levels at 30 minutes after CRH injection were significantly higher compared to cortisol levels after placebo injection (8.68 ng/ml [6.36-12.34] versus 3.43ng/ml [2.55-4.21], p<0.0001, survives Bonferroni correction) (Figure 6.3A). No correlation was found between cortisol levels at 30 minutes and the balloon volume reached at PPT (p=0.81), PTT (p=0.95).

Similar results were found in the motility study where cortisol levels were measured up to 60 minutes after CRH administration (Figure 6.3B). Compared to baseline (-15 min), an increase in salivary cortisol was seen at 30 minutes after the IV CRH injection (4.40ng/ml [2.35-5.40] versus 5.87ng/ml [5.79-6.79], p=0.0002, survives Bonferroni correction) (Figure 6.3B). No correlation was found between changes in cortisol and HRM parameters.

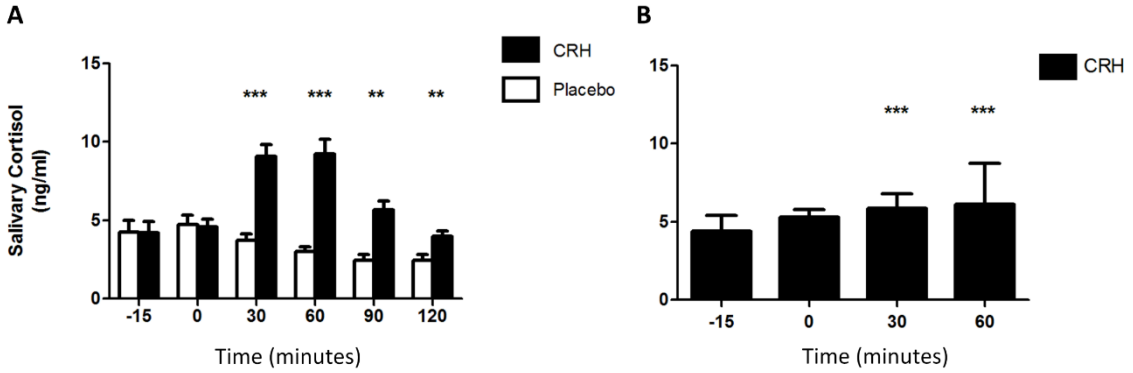

Figure 6.3 Hormonal effect of CRH administration on salivary cortisol levels. **A)** In the sensitivity study, salivary cortisol was increased at 30, 60, 90 and 120 minutes after CRH administration compared with placebo. **B)** In the motility study, cortisol levels were increased 30 and 60 minutes after administration of CRH. Median [interquartile ranges] are indicated on the graph. ***p<0.0001, **p<0.01, all significant changes survive Bonferroni correction. CRH=corticotrophin-releasing hormone.

In the sensitivity study, CRH administration exerted effects at a behavioral level. Anxiety scores were compared between CRH and placebo at the end of the procedure. No differences were found in state anxiety scores on the STAI at the end of the CRH session compared to placebo (50.00 [49.00-52.00] vs 49.50 [48.75-50.00], p=0.058). This difference could not be assessed in the motility study since baseline and CRH measurements were performed during one single procedure. However, we did not see a difference in state anxiety scores before and after CRH administration (50.00 [49.00-51.00] vs. 50.00 [50.00-51.25], p=0.4346). The POMS anxiety scores did not differ before and after the motility procedure (30.85 [26.53-41.98] vs. 31.55 [28.63-42.00], p=0.0960). When POMS anxiety scores at baseline and at the end of the sensitivity study were compared, no differences could be found between CRH or placebo conditions (6.40 [3.10-8.90] vs 3.60 [0.50-6.20], p=0.3368).

6.4 Discussion

In the current study, our aim was to elucidate the effect of exogenous CRH on esophageal sensitivity and motility. We demonstrated that IV CRH administration 1) increased salivary cortisol levels; 2) enhanced esophageal sensitivity to mechanical distention; 3) did not alter esophageal sensitivity to thermal, electrical and chemical stimulation; 4) increased esophageal contractile amplitude and decreased LES relaxation; 5) improved esophageal bolus clearance (reflected by decreased impedance ratio), increased esophageal bolus pressurization (reflected by increased IBP slope) and increased EGJ resistance to bolus flow (reflected by increased PFI).

Noxious stimuli in the esophagus are sensed by nociceptive receptors located on esophageal nerves and transmitted via spinal or vagal nerves to the central nervous system (68). Esophageal sensitivity is modulated at both peripheral and central levels. However, the details of interaction of peripheral and central factors in modulating esophageal pain perception and sensitivity have not been elucidated yet. Stressful conditions are known to increase esophageal non-peristaltic contractions (228, 235). CRH is a key mediator of responses of the body to stress and is well known to be involved in stress-related hyperalgesia. Both central and peripheral CRH signaling has been implicated in the pathogenesis of visceral hypersensitivity (236-238).

The available literature has already established a role for stress in the generation of acid-related symptoms. Fass *et al*. showed that acute auditory stress can exacerbate heartburn symptoms in GERD patients, through an enhanced perceptual response to intra-esophageal acid exposure (87). Similarly, it has been shown that stress tasks can increase subjective ratings of reflux symptoms in patients with GERD, without increasing objective parameters of acid reflux. Moreover, in patients who are chronically anxious and exposed to prolonged stress there was no habituation of reflux symptom perception upon repeated exposure to stress tasks (224). At a central level, an upregulation of central stress and arousal circuits has been postulated (239).

CRH has been implicated in the acute regulation of stress and anxiety-related behaviors and in the regulation of behavior and endocrine responses during chronic stress. Furthermore, it is well-known to mediate stress and anxiety via activation of the HPA-axis. When a stressor is

perceived, the hypothalamus will be activated to release CRH, a hypothalamic peptide which on his turn will activate the release of cortisol (121). Besides its actions on the central nervous system, also peripheral CRH signaling pathways are known to be involved in stress-related changes in GI physiology (122, 240). Larauche *et al*. stated an equally important role of the peripheral CRH signaling in visceral hypersensitivity (240). CRH is able to cross the blood barrier via a well-characterized saturating efflux system (241). Also peripheral sources of CRH have been identified: Zheng *et al.* demonstrated that eosinophils are able to express CRH in the jejunum in response to psychological stress in mice (242). Furthermore, mast cells have been shown to express CRH-receptors (240, 243, 244). CRH exerts its biological actions by interacting with CRH_1 and CRH_2 receptors (236-238, 240, 245). Genetic alterations of the CRH system have been implicated in pathophysiology of anxiety and depression (246). Preclinical and clinical data support an important role for the CRH_1 receptor in mediating acute and chronic stress-induced colonic hyperalgesia. In IBS patients, CRH may modulate visceral hypersensitivity (247). Stress induces the release of peripheral CRH which mediates the stress response of the GI tract. Hence, we used IV CRH administration to mimic this effect on esophageal sensorimotor function. CRH is able to exert physiological effects rapidly after administration (231), and the timing of procedures in the study design was based on that knowledge.

Esophageal sensitivity has been investigated in previous studies: thermal, mechanical, electrical and chemical stimuli can all be perceived in the esophagus. Since pain is a multidimensional experience, the optimal way to evaluate this sensation is to use a multimodal stimulation approach, as previously published (126, 127). We demonstrated that CRH lowered the threshold for pain perception to mechanical distention. However, we were unable to find an effect of CRH on sensitivity to thermal, electrical and chemical stimulation. These findings suggest a sensory modality-dependent effect of exogenous CRH.

While the data in the current study show that CRH mainly impacts on sensitivity to mechanical distention, it is conceivable that other sensory modalities are implicated in hypersensitive GERD patients. In a previous study it was shown that non-erosive reflux disease patients are hypersensitive to chemical, thermal and mechanical stimulation, and they react with a higher number of esophageal contractions to balloon distention compared to controls (248).

Previous studies, focusing on the colon, have shown that administration of CRH induces hypersensitivity to colorectal distention in rodents and humans (236, 238). These reports are in agreement with our findings in the esophageal sensitivity study. Nevertheless, visceral mechanosensitivity is strongly influenced by contractile activity. Hence, we used HRiM to also evaluate the impact of CRH on esophageal contractility and bolus flow (249, 250). CRH administration resulted in higher DCI values, indicating increased amplitude of esophageal contractions in response to liquid, semi-solid and solid bolus swallows. We also found an increase in IRP values for all three types of bolus consistencies, indicating reduced swallow-induced LES relaxation. These findings make it conceivable that the DCI increased as a consequence of higher IRP and indicate an increase outflow resistance, which could be the main effect of intravenous CRH administration on esophageal motility. On the other hand, the median IRP values remained within the normal range and did not exceed the cut off values for EGJ outflow obstruction (>28.28mmHg for 36 solid-state unidirectional sensors (Unisensor AG)) (135, 251). In agreement with an increased resistance at the EGJ, we could show an increased resistance to bolus flow reflected by an increase in pressure flow index (PFI). This was accompanied by higher values of IBP slope for semi-solid and solid bolus swallows, indicative of an increased degree of pressurization needed to propel the bolus onward (134). The impedance ratio for liquid and semi-solid bolus swallows, a marker for incomplete bolus transit, was decreased after administration of CRH, showing more effective bolus clearance. The findings on manometry and impedance, suggesting increased contractile tone, are in line with older studies evaluating the effects of stress on esophageal function in healthy volunteers (252, 253). Many GERD patients attribute a worsening of their symptoms to stress (87, 147, 227), by increasing contractile tone, CRH could decrease esophageal distensibility and provoke higher symptom perception in response to reflux of gastric contents into the esophagus (53). However, since we studied healthy subjects these statements should be verified in a separate study where we investigate the effect of CRH on esophageal sensitivity and motility in rGERD patients.

We acknowledge that the current study has some limitations. We did not perform dose response studies in our experiments; the choice of dose was based on available literature. Furthermore, technical limitations of the stimulation probe available at our institution prevent us from measuring the cross-sectional area of the distending balloon used for mechanical

stimulation. Therefore, we are unable to separate an effect on esophageal sensitivity from an effect on motor function, particularly esophageal compliance. Salivary cortisol levels were not maximally elevated at the time of temperature stimulation, this precludes us from fully evaluating actions of CRH through the HPA-axis on thermosensitivity.

In conclusion, we demonstrated that IV CRH administration increased esophageal sensitivity to mechanical distention in health. However, no changes were seen in sensitivity to the other stimulation modalities. Furthermore, we observed an increase in esophageal contractility and tone and a decrease in LES relaxation. As expected peripheral CRH administration increased cortisol levels. The changes in esophageal contractile properties may underlie the increased sensitivity to balloon distention after CRH.

CHAPTER 7

GENERAL DISCUSSION AND FUTURE PROSPECTS

7 General discussion and future prospects

Gastro-esophageal reflux disease (GERD) develops when the retrograde flow of gastric contents into the esophagus is causing lesions and/or troublesome symptoms. GERD is a frequent condition affecting about 20% of the adult Western population (1, 2, 5, 6). Acid suppressive therapy is highly effective in healing esophagitis but it is less efficacious in symptom control: up to 40% of patients continue to experience reflux symptoms despite acid suppressive therapy (54-56). These patients are referred to as refractory GERD (rGERD)**.** A range of underlying mechanisms to explain PPI-resistant symptoms in patients with rGERD have already been proposed: persistent volume reflux, the composition of the refluxed gastric contents (weakly acidic and non-acid reflux, gas reflux), and the proximal extent of reflux events (60). In addition, also visceral hypersensitivity is known to play a role in the generation of reflux symptoms. Furthermore, it has been demonstrated that psychological factors might influence the perception of heartburn in rGERD patients (86).

We hypothesize that an increased esophageal sensitivity is crucially involved in symptom generation and symptom perception in rGERD patients on PPI therapy. Changes in esophageal integrity and altered signaling of descending anti-nociceptive pathways could be a mechanism behind this hypersensitivity. The overall objective of this PhD project was to obtain a better understanding in the mechanisms determining the generation and the perception of symptoms in patients with rGERD on PPI therapy.

7.1 Involvement of esophageal sensitivity and esophageal integrity in symptom perception in refractory GERD

In Chapter 4 we investigated why rGERD patients continue to experience symptoms of GERD while on a double dose of PPI. As mentioned above, perception of reflux and generation of symptoms is influenced by several factors. In this study we demonstrated that altered reflux parameters play a pivotal role: the number of non-acid reflux events, the number of reflux events with a high proximal extent and volume exposure were higher in rGERD on PPI compared to HV. Furthermore, the distribution of mixed reflux and liquid reflux events was different in rGERD patients and HV. These findings confirm previous data that volume reflux and proximal reflux events are potentially involved in symptom generation in patients refractory to PPI therapy (151). Although we could not completely reproduce data from a previous study of our group where it was shown that rGERD patients display an increased

sensitivity to multimodal esophageal stimulation (193), we confirmed the presence of an increased esophageal sensitivity to acid infusion and electrical stimulation. Patients with rGERD symptoms in our study were more sensitive to chemical stimulation compared to HV despite acid suppressive therapy. We conclude that this may indicate peripheral sensitization of acid sensitive receptors present in the esophageal wall (52, 119). The recurrent presence of acidic gastric contents may have caused sensitization of chemoreceptors and thereby lead to esophageal hypersensitivity to acid. However, the presence of acid is not the only factor involved in triggering symptoms since the majority of patients did not have a positive association between symptoms and acid reflux when studies on a double dose of PPI. The increased sensitivity to acid infusion may therefore be a marker of a broader sensitization allowing generation of GERD symptoms in response to a number of different events (non-acid reflux, distention, contraction, *etc*).

Besides volume reflux and esophageal hypersensitivity, an impaired esophageal epithelial integrity has also been postulated to underlie rGERD symptoms (52, 78). In our study we could not differentiate between rGERD patients on a double dose of PPI and HV based on measurements of transepithelial electrical resistance (TEER) and esophageal permeability measured by passage of fluorescein. We observed a substantial overlap in TEER and passage of fluorescein between rGERD and HV and could not confirm the hypothesis that alterations in epithelial integrity are a candidate mechanism underlying esophageal sensitivity changes in rGERD patients on a double dose of PPI treatment. In addition, when esophageal integrity was assessed *in vivo* by impedance baseline values, we found that impedance baseline was significantly higher in rGERD patients on PPI compared to HV at 5 and 15cm above the LES. Since it has been shown that PPI treatment can increase impedance baseline values in a subgroup of HV (161), we concluded that the observed differences could be attributed to the fact that HV were not on PPI treatment while rGERD patients were on a double dose of PPI.

Finally, we demonstrated that in patients with rGERD symptoms, feelings of anxiety and negative affect were more present compared to HV. Anxiety scores measured by the STAI state anxiety questionnaire decreased in HV after the multimodal stimulation test, while in rGERD patients STAI scores remained similar before and after the stimulation. Positive affect scores tended to be lower in rGERD compared to HV and negative affect score was significantly higher in rGERD. These findings are in agreement with available literature stating that negative

emotion and anxiety may have implications on the perception of GERD symptoms (86, 89, 159).

It can be argued that the comparison between rGERD patients and healthy controls is a limitation of our experimental design. However, in our studies, in general we always choose a two-step approach with initial explorations in healthy controls before studying patients. A first step in unraveling the underlying pathophysiology of refractory GERD was to compare reflux characteristics, esophageal sensitivity and esophageal integrity in a normal situation *i.e.* healthy asymptomatic controls with patients with rGERD symptoms. A second step would be a comparison between patients that respond to PPI therapy and rGERD patients. In the end this should give us a better insight in the specific aspects where rGERD patients differ from PPI responsive GERD patients. For future projects we will further increase the sample size of our rGERD cohort in order to compare data on esophageal sensitivity, 24 hour MII-pH monitoring and esophageal integrity in different subgroups of rGERD including NERD, reflux hypersensitive patients and patients with functional heartburn. In addition, we consider it useful to further investigate if an impaired esophageal integrity does play a role in rGERD symptoms. Dilated intercellular spaces (DIS) are reported to be an accurate morphological marker in GERD, reflecting the alteration of esophageal epithelial integrity (79, 119). Heartburn perception has been associated with presence of DIS in the esophageal epithelium (82, 83), indicating that acid and other gastric compounds could trigger nerve endings which are present between cells of the lower layers of the epithelium. Therefore, transmission electron microscopy will be performed on proximal and distal esophageal biopsies of HV and rGERD patients on PPI to investigate if DIS are indeed present and could underlie sensitivity changes that were observed during multimodal esophageal stimulation. Another factor involved in esophageal sensations is the up-regulation of acid sensing receptors mainly located on the nerve endings present in the esophageal mucosa (254, 255)**.** We already showed preliminary data of Western blot experiments on the presence of PAR-2, ASCIC3 and δENaC receptors in rGERD patients. However, additional immunofluorescence and PCR analysis experiments will be performed to further quantify if acid sensitive receptors are upregulated in rGERD compared to HV. In addition, since MII-pH monitoring in rGERD patients on PPI demonstrated that non-acid reflux seems to be involved in symptom perception, a future consideration could be to perform an additional chemical stimulation test with a non-acid

solution instead of hydrochloric acid. A solution containing bile salts could also be used for this non-acid stimulation. Furthermore, the composition of bile salt content and composition in gastric juice should be compared between rGERD on PPI and HV.

7.2 The effect of blocking anti-nociceptive pathways on esophageal sensitivity in health

Over the last two decades, brain areas involved in pain perception have been identified, however the neurophysiologic basis for the development of visceral hypersensitivity remains elusive. There is evidence for changes in descending anti-nociceptive pathways as a mechanism underlying increased esophageal sensitivity (256, 257). In order to advance knowledge on the role of esophageal hypersensitivity in patients with rGERD, we investigated the effect of blocking three anti-nociceptive pathways on esophageal sensation in HV: the endogenous opioid system, the serotonin system, and the dopamine system **(Chapter 5)**. Esophageal sensitivity was assessed using a multimodal stimulation model.

The major findings of this project were: i) peripheral and centrally acting μ-opioid antagonists did not alter esophageal sensitivity to multimodal stimulation in HV. ii) ATD increased sensitivity to acid infusion, suggesting that the serotonin system is a candidate target for treatment of esophageal hypersensitivity. Furthermore, gender differences were observed during the multimodal stimulation protocol in both the placebo and ATD condition. iii) Blocking the D_2 receptor by IV administration of chlorpromazine did not have an influence on esophageal sensitivity to multimodal stimulation in health. Chlorpromazine did have an effect on mood in HV, positive affect scores were significantly lower after the stimulation test compared to before stimulation.

When the overall effect of blocking these three types of anti-nociceptive pathways in healthy volunteers was evaluated, we concluded that the results of the ATD study will have the most significant impact on the future treatment of rGERD patients. The results of this experiment clearly show the potential benefit of so called ‘neuromodulators’ including SSRIs in the treatment of reflux hypersensitive patients and patients with functional heartburn. ATD demonstrated that low levels of serotonin are likely to be involved in increased acid sensitivity. Our data confirm the results of earlier studies that proposed to use SSRIs such as citalopram in the treatment of esophageal hypersensitivity (115, 258). In future projects we will consider

to perform a multimodal stimulation test with an SSRI *e.g.* citalopram in patients with rGERD on PPI treatment. Our group already performed a multimodal stimulation test after administration of citalopram in HV and it was shown that this had no effect on esophageal stimulation. However our results from the ATD study suggest that in cases of low serotonin the outcome could be different. In addition, we will measure plasma levels of tryptophan in rGERD patients that are willing to participate in the multimodal stimulation study to evaluate if levels of TRP are altered in comparison with HV.

In this project we already investigated three neurotransmitter systems. Future experiments should additionally focus on the role of the endocannabinoid system in modulation of esophageal sensation. Endocannabinoids can be released by postsynaptic neurons and diffuse to nerve terminals where they reduce transmitter release, in this way they can function as retrograde messengers (259, 260). Animal studies provide evidence for the involvement of the endocannabinoid system in visceral pain perception (261-263). A study of our group showed that rimonabant, a CB_1 receptor antagonist, enhances postprandial LES pressure and decreases transient lower esophageal sphincter relaxations (TLESRs) in HV (264). Currently it is not completely clear whether endogenous ligands of the endocannabinoid system are involved in the control of esophageal function. Therefore**,** in a future study we will investigate the influence of the cannabinoid agonist dronabinol on esophageal sensitivity in HV by using the multimodal stimulation protocol. In conclusion, one of the limitations of the multimodal esophageal stimulation protocol was the occurrence of a ceiling effect during the mechanical and in some cases in chemical stimulation. For future studies in HV it could be useful to perform a screening visit to verify whether HV reach the sensitivity thresholds at maximal balloon volume and acid infusion. When they reach the sensitivity thresholds within the limits of the stimulation tests they can proceed with the study.

7.3 Intravenous administration of corticotropin-releasing hormone affects esophageal mechanosensitivity and alters esophageal motility in health

Stressful conditions are known to increase esophageal non-peristaltic contractions and are known to be involved in modulation of symptom perception in rGERD (228, 235). Stress induces the release of peripheral corticotropin-releasing hormone (CRH) which mediates the stress response of the GI tract. CRH is well known to be involved in stress-related hyperalgesia, and has been implicated in the pathogenesis of visceral hypersensitivity (236-238).

In **Chapter 6**, our aim was to elucidate the effect of exogenous CRH on esophageal sensitivity and motility. We demonstrated that IV CRH administration i) increased salivary cortisol levels; ii) enhanced esophageal sensitivity to mechanical distention; iii) did not alter esophageal sensitivity to thermal, electrical and chemical stimulation; iv) increased esophageal contractile amplitude and decreased LES relaxation; v) improved esophageal bolus clearance (reflected by decreased impedance ratio), increased esophageal bolus pressurization (reflected by increased IBP slope) and increased EGJ resistance to bolus flow (reflected by increased PFI).
Our findings in HV are of particular interest since it has been shown that many GERD patients report a worsening of their symptoms due to stress (87, 147, 227). By increasing contractile tone, CRH could decrease esophageal distensibility and provoke higher symptom perception in response to reflux of gastric contents into the esophagus (53). For future projects we will measure cortisol levels in saliva of rGERD patient to further investigate if the same statements that were made based on our findings in HV apply to rGERD patients. Furthermore, we will consider to optimize our multimodal stimulation catheter in order to be able to measure impedance and pressure changes simultaneously with the assessment of esophageal sensitivity. In the current study due to technical limitations of the stimulation probe available at our institution we are unable to separate an effect on esophageal sensitivity from an effect on motor function, particularly esophageal compliance.

7.4 General Conclusion

In this PhD project we have investigated underlying mechanisms of ongoing symptoms of GERD despite acid suppressive therapy. After characterization of reflux parameters in rGERD on PPI therapy we found that ongoing non-acid reflux, volume exposure and the proximal extent of reflux events are involved in symptom generation in rGERD. Multimodal esophageal stimulation revealed that rGERD patients have an increased esophageal sensitivity to electrical and chemical stimulation indicating that an increased acid sensitivity may contribute to symptom perception in these patients. In rGERD patient on a double dose of PPI, impaired esophageal epithelial integrity measured *in vitro* and *in vivo* did not explain this increased sensitivity since no changes in esophageal integrity were present when our rGERD cohort was compared to HV. The serotonin system seems to be a useful target for future therapies in patients with reflux hypersensitivity and functional heartburn since low levels of serotonin are related to increased acid sensitivity. Psychosocial stressors have been demonstrated to be

important in modulation of esophageal sensation, in agreement with this finding we found that the stress hormone CRH was able to increase esophageal sensitivity and alter esophageal motility.

In conclusion, rGERD is a complex spectrum disease with a broad range of pathological consequences. More research is needed to enhance our knowledge concerning PPI failure in patients with refractory symptoms of GERD. A better insight in factors driving symptom perception along the GERD spectrum is imperative to develop novel treatment strategies that are better tailored to the individual patient. This could be achieved by identifying different mechanisms in the distinct subgroups of patients with symptoms of GERD.

CHAPTER 8

REFERENCES

8 References

1. Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R, Global Consensus G. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. The American journal of gastroenterology. 2006;101(8):1900-20; quiz 43.
2. El-Serag HB, Sweet S, Winchester CC, Dent J. Update on the epidemiology of gastro-oesophageal reflux disease: a systematic review. Gut. 2014;63(6):871-80.
3. Jaspersen D, Kulig M, Labenz J, Leodolter A, Lind T, Meyer-Sabellek W, et al. Prevalence of extra-oesophageal manifestations in gastro-oesophageal reflux disease: an analysis based on the ProGERD Study. Alimentary pharmacology & therapeutics. 2003;17(12):1515-20.
4. Locke GR, 3rd, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ, 3rd. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. Gastroenterology. 1997;112(5):1448-56.
5. Spechler SJ. Epidemiology and natural history of gastro-oesophageal reflux disease. Digestion. 1992;51 Suppl 1:24-9.
6. Ronkainen J, Aro P, Storskrubb T, Johansson SE, Lind T, Bolling-Sternevald E, et al. High prevalence of gastroesophageal reflux symptoms and esophagitis with or without symptoms in the general adult Swedish population: a Kalixanda study report. Scand J Gastroenterol. 2005;40(3):275-85.
7. Boeckxstaens GE, Rohof WO. Pathophysiology of gastroesophageal reflux disease. Gastroenterol Clin North Am. 2014;43(1):15-25.
8. Ang D, Sifrim D, Tack J. Mechanisms of heartburn. Nat Clin Pract Gastroenterol Hepatol. 2008;5(7):383-92.
9. Dent J. Barrett's esophagus: A historical perspective, an update on core practicalities and predictions on future evolutions of management. J Gastroenterol Hepatol. 2011;26 Suppl 1:11-30.
10. Ronkainen J, Aro P, Storskrubb T, Lind T, Bolling-Sternevald E, Junghard O, et al. Gastro-oesophageal reflux symptoms and health-related quality of life in the adult general population--the Kalixanda study. Alimentary pharmacology & therapeutics. 2006;23(12):1725-33.
11. Kusano M, Shimoyama Y, Sugimoto S, Kawamura O, Maeda M, Minashi K, et al. Development and evaluation of FSSG: frequency scale for the symptoms of GERD. J Gastroenterol. 2004;39(9):888-91.
12. Ducrotté P, Zerbib F. ReQuest: a new questionnaire for the simultaneous evaluation of symptoms and well-being in patients with gastro-oesophageal reflux. Digestion. 2007;75 Suppl 1:79-86.
13. Jonasson C, Wernersson B, Hoff DA, Hatlebakk JG. Validation of the GerdQ questionnaire for the diagnosis of gastro-oesophageal reflux disease. Aliment Pharmacol Ther. 2013;37(5):564-72.
14. Shay SS, Bomeli S, Richter J. Multichannel intraluminal impedance accurately detects fasting, recumbent reflux events and their clearing. Am J Physiol Gastrointest Liver Physiol. 2002;283(2):G376-83.
15. Bredenoord AJ, Pandolfino JE, Smout AJ. Gastro-oesophageal reflux disease. Lancet. 2013;381(9881):1933-42.
16. Scarpellini E, Ang D, Pauwels A, De Santis A, Vanuytsel T, Tack J. Management of refractory typical GERD symptoms. Nat Rev Gastroenterol Hepatol. 2016;13(5):281-94.
17. Vaezi MF, Schroeder PL, Richter JE. Reproducibility of proximal probe pH parameters in 24-hour ambulatory esophageal pH monitoring. The American journal of gastroenterology. 1997;92(5):825-9.
18. Bhat YM, McGrath KM, Bielefeldt K. Wireless esophageal pH monitoring: new technique means new questions. Journal of clinical gastroenterology. 2006;40(2):116-21.
19. Zerbib F, Roman S, Ropert A, des Varannes SB, Pouderoux P, Chaput U, et al. Esophageal pH-impedance monitoring and symptom analysis in GERD: a study in patients off and on therapy. Am J Gastroenterol. 2006;101(9):1956-63.

20. Zerbib F, Roman S, Bruley Des Varannes S, Gourcerol G, Coffin B, Ropert A, et al. Normal values of pharyngeal and esophageal 24-hour pH impedance in individuals on and off therapy and interobserver reproducibility. Clin Gastroenterol Hepatol. 2013;11(4):366-72.
21. Shay S, Tutuian R, Sifrim D, Vela M, Wise J, Balaji N, et al. Twenty-four hour ambulatory simultaneous impedance and pH monitoring: a multicenter report of normal values from 60 healthy volunteers. Am J Gastroenterol. 2004;99(6):1037-43.
22. Roman S, Gyawali CP, Savarino E, Yadlapati R, Zerbib F, Wu J, et al. Ambulatory reflux monitoring for diagnosis of gastro-esophageal reflux disease: Update of the Porto consensus and recommendations from an international consensus group. Neurogastroenterol Motil. 2017;29(10):1-15.
23. Savarino E, Zentilin P, Mastracci L, Dulbecco P, Marabotto E, Gemignani L, et al. Microscopic esophagitis distinguishes patients with non-erosive reflux disease from those with functional heartburn. J Gastroenterol. 2013;48(4):473-82.
24. Calabrese C, Bortolotti M, Fabbri A, Areni A, Cenacchi G, Scialpi C, et al. Reversibility of GERD ultrastructural alterations and relief of symptoms after omeprazole treatment. Am J Gastroenterol. 2005;100(3):537-42.
25. Numans ME, Lau J, de Wit NJ, Bonis PA. Short-term treatment with proton-pump inhibitors as a test for gastroesophageal reflux disease: a meta-analysis of diagnostic test characteristics. Ann Intern Med. 2004;140(7):518-27.
26. Hayat JO, Gabieta-Somnez S, Yazaki E, Kang JY, Woodcock A, Dettmar P, et al. Pepsin in saliva for the diagnosis of gastro-oesophageal reflux disease. Gut. 2015;64(3):373-80.
27. Mikami DJ, Murayama KM. Physiology and pathogenesis of gastroesophageal reflux disease. Surg Clin North Am. 2015;95(3):515-25.
28. Mittal RK, Balaban DH. The esophagogastric junction. N Engl J Med. 1997;336(13):924-32.
29. Holloway RH. The anti-reflux barrier and mechanisms of gastro-oesophageal reflux. Baillieres Best Pract Res Clin Gastroenterol. 2000;14(5):681-99.
30. Mittal RK, Holloway RH, Penagini R, Blackshaw LA, Dent J. Transient lower esophageal sphincter relaxation. Gastroenterology. 1995;109(2):601-10.
31. Sifrim D, Holloway R. Transient lower esophageal sphincter relaxations: how many or how harmful? Am J Gastroenterol. 2001;96(9):2529-32.
32. Trudgill NJ, Riley SA. Transient lower esophageal sphincter relaxations are no more frequent in patients with gastroesophageal reflux disease than in asymptomatic volunteers. Am J Gastroenterol. 2001;96(9):2569-74.
33. Sloan S, Kahrilas PJ. Impairment of esophageal emptying with hiatal hernia. Gastroenterology. 1991;100(3):596-605.
34. Kahrilas PJ, Lin S, Chen J, Manka M. The effect of hiatus hernia on gastro-oesophageal junction pressure. Gut. 1999;44(4):476-82.
35. Jones MP, Sloan SS, Rabine JC, Ebert CC, Huang CF, Kahrilas PJ. Hiatal hernia size is the dominant determinant of esophagitis presence and severity in gastroesophageal reflux disease. Am J Gastroenterol. 2001;96(6):1711-7.
36. Karvelis KC, Drane WE, Johnson DA, Silverman ED. Barrett esophagus: decreased esophageal clearance shown by radionuclide esophageal scintigraphy. Radiology. 1987;162(1 Pt 1):97-9.
37. Singh P, Adamopoulos A, Taylor RH, Colin-Jones DG. Oesophageal motor function before and after healing of oesophagitis. Gut. 1992;33(12):1590-6.
38. Savarino E, Gemignani L, Pohl D, Zentilin P, Dulbecco P, Assandri L, et al. Oesophageal motility and bolus transit abnormalities increase in parallel with the severity of gastro-oesophageal reflux disease. Aliment Pharmacol Ther. 2011;34(4):476-86.
39. Lemme EM, Abrahão-Junior LJ, Manhães Y, Shechter R, Carvalho BB, Alvariz A. Ineffective esophageal motility in gastroesophageal erosive reflux disease and in nonerosive reflux disease: are they different? J Clin Gastroenterol. 2005;39(3):224-7.

40. Hong SJ, Ko BM, Jung IS, Ryu CB, Moon JH, Cho JY, et al. Relevance of ineffective esophageal motility and hyperactive acid sensitization in patients with gastroesophageal reflux. J Gastroenterol Hepatol. 2007;22(10):1662-5.
41. Penagini R, Bravi I. The role of delayed gastric emptying and impaired oesophageal body motility. Best Pract Res Clin Gastroenterol. 2010;24(6):831-45.
42. Anand G, Katz PO. Gastroesophageal reflux disease and obesity. Gastroenterol Clin North Am. 2010;39(1):39-46.
43. El-Serag HB, Hashmi A, Garcia J, Richardson P, Alsarraj A, Fitzgerald S, et al. Visceral abdominal obesity measured by CT scan is associated with an increased risk of Barrett's oesophagus: a case-control study. Gut. 2014;63(2):220-9.
44. Lee YY, Wirz AA, Whiting JG, Robertson EV, Smith D, Weir A, et al. Waist belt and central obesity cause partial hiatus hernia and short-segment acid reflux in asymptomatic volunteers. Gut. 2014;63(7):1053-60.
45. Fletcher J, Wirz A, Young J, Vallance R, McColl KE. Unbuffered highly acidic gastric juice exists at the gastroesophageal junction after a meal. Gastroenterology. 2001;121(4):775-83.
46. Mitchell DR, Derakhshan MH, Robertson EV, McColl KE. The Role of the Acid Pocket in Gastroesophageal Reflux Disease. J Clin Gastroenterol. 2016;50(2):111-9.
47. Beaumont H, Bennink RJ, de Jong J, Boeckxstaens GE. The position of the acid pocket as a major risk factor for acidic reflux in healthy subjects and patients with GORD. Gut. 2010;59(4):441-51.
48. Emerenziani S, Sifrim D. Gastroesophageal reflux and gastric emptying, revisited. Curr Gastroenterol Rep. 2005;7(3):190-5.
49. Szczesniak MM, Fuentealba SE, Cook IJ. Acid sensitization of esophageal mucosal afferents: implication for symptom perception in patients across the gastroesophageal reflux disease spectrum. Clin J Pain. 2013;29(1):70-7.
50. Kondo T, Oshima T, Tomita T, Fukui H, Watari J, Okada H, et al. Prostaglandin E(2) mediates acid-induced heartburn in healthy volunteers. Am J Physiol Gastrointest Liver Physiol. 2013;304(6):G568-73.
51. Kondo T, Oshima T, Tomita T, Fukui H, Okada H, Watari J, et al. The Nonsteroidal Anti-inflammatory Drug Diclofenac Reduces Acid-Induced Heartburn Symptoms in Healthy Volunteers. Clin Gastroenterol Hepatol. 2015;13(7):1249-55.e1.
52. Weijenborg PW, Smout AJ, Verseijden C, van Veen HA, Verheij J, de Jonge WJ, et al. Hypersensitivity to acid is associated with impaired esophageal mucosal integrity in patients with gastroesophageal reflux disease with and without esophagitis. Am J Physiol Gastrointest Liver Physiol. 2014;307(3):G323-9.
53. Yang M, Li ZS, Chen DF, Zou DW, Xu XR, Fang DC, et al. Quantitative assessment and characterization of visceral hyperalgesia evoked by esophageal balloon distention and acid perfusion in patients with functional heartburn, nonerosive reflux disease, and erosive esophagitis. Clin J Pain. 2010;26(4):326-31.
54. Edwards SJ, Lind T, Lundell L. Systematic review: proton pump inhibitors (PPIs) for the healing of reflux oesophagitis - a comparison of esomeprazole with other PPIs. Alimentary pharmacology & therapeutics. 2006;24(5):743-50.
55. Fass R, Shapiro M, Dekel R, Sewell J. Systematic review: proton-pump inhibitor failure in gastro-oesophageal reflux disease--where next? Aliment Pharmacol Ther. 2005;22(2):79-94.
56. Tutuian R, Vela MF, Hill EG, Mainie I, Agrawal A, Castell DO. Characteristics of symptomatic reflux episodes on Acid suppressive therapy. The American journal of gastroenterology. 2008;103(5):1090-6.
57. Bredenoord AJ, Smout AJ. Refractory gastrooesophageal reflux disease. Eur J Gastroenterol Hepatol. 2008;20(3):217-23.
58. Sifrim D, Mittal R, Fass R, Smout A, Castell D, Tack J, et al. Review article: acidity and volume of the refluxate in the genesis of gastro-oesophageal reflux disease symptoms. Aliment Pharmacol Ther. 2007;25(9):1003-17.

59. Aziz Q, Fass R, Gyawali CP, Miwa H, Pandolfino JE, Zerbib F. Functional Esophageal Disorders. Gastroenterology. 2016;150:1368-79.
60. Fass R, Sifrim D. Management of heartburn not responding to proton pump inhibitors. Gut. 2009;58(2):295-309.
61. Herregods TV, Troelstra M, Weijenborg PW, Bredenoord AJ, Smout AJ. Patients with refractory reflux symptoms often do not have GERD. Neurogastroenterol Motil. 2015;27(9):1267-73.
62. Woodland P, Sifrim D, Krarup AL, Brock C, Frøkjaer JB, Lottrup C, et al. The neurophysiology of the esophagus. Ann N Y Acad Sci. 2013;1300:53-70.
63. Knowles CH, Aziz Q. Basic and clinical aspects of gastrointestinal pain. Pain. 2009;141(3):191-209.
64. Brock C, Gregersen H, Gyawali CP, Lottrup C, Furnari M, Savarino E, et al. The sensory system of the esophagus--what do we know? Ann N Y Acad Sci. 2016;1380(1):91-103.
65. Knowles CH, Aziz Q. Visceral hypersensitivity in non-erosive reflux disease. Gut. 2008;57(5):674-83.
66. Trimble KC, Pryde A, Heading RC. Lowered oesophageal sensory thresholds in patients with symptomatic but not excess gastro-oesophageal reflux: evidence for a spectrum of visceral sensitivity in GORD. Gut. 1995;37(1):7-12.
67. Boecxstaens V, Pauwels A, Blondeau K, Oustamanolakis P, Altan E, Boeckxstaens G, et al. Refractory GERD patients display increased visceral hypersensitivity for thermal, chemical and mechanical esophageal stimulation. Digestive Disease Week; Orlando: Gastroenterology; 2013. p. S-936.
68. Miwa H, Kondo T, Oshima T, Fukui H, Tomita T, Watari J. Esophageal sensation and esophageal hypersensitivity - overview from bench to bedside. J Neurogastroenterol Motil. 2010;16(4):353-62.
69. Benham CD, Davis JB, Randall AD. Vanilloid and TRP channels: a family of lipid-gated cation channels. Neuropharmacology. 2002;42(7):873-88.
70. Bhat YM, Bielefeldt K. Capsaicin receptor (TRPV1) and non-erosive reflux disease. Eur J Gastroenterol Hepatol. 2006;18(3):263-70.
71. Bielefeldt K, Davis BM. Differential effects of ASIC3 and TRPV1 deletion on gastroesophageal sensation in mice. Am J Physiol Gastrointest Liver Physiol. 2008;294(1):G130-8.
72. Fujino K, de la Fuente SG, Takami Y, Takahashi T, Mantyh CR. Attenuation of acid induced oesophagitis in VR-1 deficient mice. Gut. 2006;55(1):34-40.
73. Yoshida N, Kuroda M, Suzuki T, Kamada K, Uchiyama K, Handa O, et al. Role of nociceptors/neuropeptides in the pathogenesis of visceral hypersensitivity of nonerosive reflux disease. Dig Dis Sci. 2013;58(8):2237-43.
74. Kim JJ, Kim N, Choi YJ, Kim JS, Jung HC. Increased TRPV1 and PAR2 mRNA expression levels are associated only with the esophageal reflux symptoms, but not with the extraesophageal reflux symptoms. Medicine (Baltimore). 2016;95(32):e4387.
75. Page AJ, Brierley SM, Martin CM, Price MP, Symonds E, Butler R, et al. Different contributions of ASIC channels 1a, 2, and 3 in gastrointestinal mechanosensory function. Gut. 2005;54(10):1408-15.
76. Wu L, Oshima T, Shan J, Sei H, Tomita T, Ohda Y, et al. PAR-2 activation enhances weak acid-induced ATP release through TRPV1 and ASIC sensitization in human esophageal epithelial cells. Am J Physiol Gastrointest Liver Physiol. 2015;309(8):G695-702.
77. Yamamura H, Ugawa S, Ueda T, Nagao M, Joh T, Shimada S. Epithelial Na+ channel delta subunit is an acid sensor in the human oesophagus. Eur J Pharmacol. 2008;600(1-3):32-6.
78. Farré R. Pathophysiology of gastro-esophageal reflux disease: a role for mucosa integrity? Neurogastroenterol Motil. 2013;25(10):783-99.
79. van Malenstein H, Farré R, Sifrim D. Esophageal dilated intercellular spaces (DIS) and nonerosive reflux disease. Am J Gastroenterol. 2008;103(4):1021-8.
80. Hom C, Vaezi MF. Extraesophageal manifestations of gastroesophageal reflux disease. Gastroenterol Clin North Am. 2013;42(1):71-91.
81. Jovov B, Que J, Tobey NA, Djukic Z, Hogan BL, Orlando RC. Role of E-cadherin in the pathogenesis of gastroesophageal reflux disease. Am J Gastroenterol. 2011;106(6):1039-47.

82. Caviglia R, Ribolsi M, Gentile M, Rabitti C, Emerenziani S, Guarino MP, et al. Dilated intercellular spaces and acid reflux at the distal and proximal oesophagus in patients with non-erosive gastro-oesophageal reflux disease. Alimentary pharmacology & therapeutics. 2007;25(5):629-36.
83. Cicala M, Emerenziani S, Caviglia R, Guarino MP, Vavassori P, Ribolsi M, et al. Intra-oesophageal distribution and perception of acid reflux in patients with non-erosive gastro-oesophageal reflux disease. Alimentary pharmacology & therapeutics. 2003;18(6):605-13.
84. Aro P, Talley NJ, Ronkainen J, Storskrubb T, Vieth M, Johansson SE, et al. Anxiety is associated with uninvestigated and functional dyspepsia (Rome III criteria) in a Swedish population-based study. Gastroenterology. 2009;137(1):94-100.
85. Van Oudenhove L, Vandenberghe J, Geeraerts B, Vos R, Persoons P, Demyttenaere K, et al. Relationship between anxiety and gastric sensorimotor function in functional dyspepsia. Psychosom Med. 2007;69(5):455-63.
86. Johnston BT, Lewis SA, Love AH. Psychological factors in gastro-oesophageal reflux disease. Gut. 1995;36(4):481-2.
87. Fass R, Naliboff BD, Fass SS, Peleg N, Wendel C, Malagon IB, et al. The effect of auditory stress on perception of intraesophageal acid in patients with gastroesophageal reflux disease. Gastroenterology. 2008;134(3):696-705.
88. Johnston BT, Lewis SA, Collins JS, McFarland RJ, Love AH. Acid perception in gastro-oesophageal reflux disease is dependent on psychosocial factors. Scand J Gastroenterol. 1995;30(1):1-5.
89. Kessing BF, Bredenoord AJ, Saleh CM, Smout AJ. Effects of anxiety and depression in patients with gastroesophageal reflux disease. Clin Gastroenterol Hepatol. 2015;13(6):1089-95.e1.
90. Blondeau K PA, Boecxstaens V, Caenepeel C, Depeyper S, Holvoet L, Boeckxstaens GE, Tack, JF, Van Oudenhove L. Affective disorders, somatization and body awareness are associated with positive symptom association during 24 hour impedance-pH monitoring in patients with reflux symptoms. gastroenteroly. 2012;142(5):1:S204-S Digestive Disease Week, San Diego.
91. Koek GH, Sifrim D, Lerut T, Janssens J, Tack J. Effect of the GABA(B) agonist baclofen in patients with symptoms and duodeno-gastro-oesophageal reflux refractory to proton pump inhibitors. Gut. 2003;52(10):1397-402.
92. Vela MF, Tutuian R, Katz PO, Castell DO. Baclofen decreases acid and non-acid post-prandial gastro-oesophageal reflux measured by combined multichannel intraluminal impedance and pH. Aliment Pharmacol Ther. 2003;17(2):243-51.
93. Lidums I, Lehmann A, Checklin H, Dent J, Holloway RH. Control of transient lower esophageal sphincter relaxations and reflux by the GABA(B) agonist baclofen in normal subjects. Gastroenterology. 2000;118(1):7-13.
94. Vakil NB, Huff FJ, Cundy KC. Randomised clinical trial: arbaclofen placarbil in gastro-oesophageal reflux disease--insights into study design for transient lower sphincter relaxation inhibitors. Aliment Pharmacol Ther. 2013;38(2):107-17.
95. Shaheen NJ, Denison H, Björck K, Karlsson M, Silberg DG. Efficacy and safety of lesogaberan in gastro-oesophageal reflux disease: a randomised controlled trial. Gut. 2013;62(9):1248-55.
96. Frisby CL, Mattsson JP, Jensen JM, Lehmann A, Dent J, Blackshaw LA. Inhibition of transient lower esophageal sphincter relaxation and gastroesophageal reflux by metabotropic glutamate receptor ligands. Gastroenterology. 2005;129(3):995-1004.
97. Tack J, Camilleri M, Chang L, Chey WD, Galligan JJ, Lacy BE, et al. Systematic review: cardiovascular safety profile of 5-HT(4) agonists developed for gastrointestinal disorders. Aliment Pharmacol Ther. 2012;35(7):745-67.
98. Zaninotto G, Attwood SE. Surgical management of refractory gastro-oesophageal reflux. Br J Surg. 2010;97(2):139-40.
99. Wykypiel H, Wetscher GJ, Klingler P, Glaser K. The Nissen fundoplication: indication, technical aspects and postoperative outcome. Langenbecks Arch Surg. 2005;390(6):495-502.

100. Galmiche JP, Hatlebakk J, Attwood S, Ell C, Fiocca R, Eklund S, et al. Laparoscopic antireflux surgery vs esomeprazole treatment for chronic GERD: the LOTUS randomized clinical trial. JAMA. 2011;305(19):1969-77.
101. Tian ZC, Wang B, Shan CX, Zhang W, Jiang DZ, Qiu M. A Meta-Analysis of Randomized Controlled Trials to Compare Long-Term Outcomes of Nissen and Toupet Fundoplication for Gastroesophageal Reflux Disease. PLoS One. 2015;10(6):e0127627.
102. Du X, Wu JM, Hu ZW, Wang F, Wang ZG, Zhang C, et al. Laparoscopic Nissen (total) versus anterior 180° fundoplication for gastro-esophageal reflux disease: A meta-analysis and systematic review. Medicine (Baltimore). 2017;96(37):e8085.
103. Lundell L, Bell M, Ruth M. Systematic review: laparoscopic fundoplication for gastroesophageal reflux disease in partial responders to proton pump inhibitors. World J Gastroenterol. 2014;20(3):804-13.
104. Anvari M, Allen C. Surgical outcome in gastro-esophageal reflux disease patients with inadequate response to proton pump inhibitors. Surg Endosc. 2003;17(7):1029-35.
105. Hamdy E, El Nakeeb A, Hamed H, El Hemaly M, ElHak NG. Outcome of laparoscopic Nissen fundoplication for gastroesophageal reflux disease in non-responders to proton pump inhibitors. J Gastrointest Surg. 2014;18(9):1557-62.
106. Wilkerson PM, Stratford J, Jones L, Sohanpal J, Booth MI, Dehn TC. A poor response to proton pump inhibition is not a contraindication for laparoscopic antireflux surgery for gastro esophageal reflux disease. Surg Endosc. 2005;19(9):1272-7.
107. Pandolfino JE. The Use of Endoscopy and Radiofrequency Ablation for the Treatment of GERD. Gastroenterol Hepatol (N Y). 2015;11(12):847-9.
108. Azagury D, Morton J. Surgical Anti-Reflux Options Beyond Fundoplication. Curr Gastroenterol Rep. 2017;19(7):35.
109. Rodríguez L, Rodriguez P, Gómez B, Ayala JC, Saba J, Perez-Castilla A, et al. Electrical stimulation therapy of the lower esophageal sphincter is successful in treating GERD: final results of open-label prospective trial. Surg Endosc. 2013;27(4):1083-92.
110. Rohof WO, Bennink RJ, Smout AJ, Thomas E, Boeckxstaens GE. An alginate-antacid formulation localizes to the acid pocket to reduce acid reflux in patients with gastroesophageal reflux disease. Clin Gastroenterol Hepatol. 2013;11(12):1585-91; quiz e90.
111. Reimer C, Lødrup AB, Smith G, Wilkinson J, Bytzer P. Randomised clinical trial: alginate (Gaviscon Advance) vs. placebo as add-on therapy in reflux patients with inadequate response to a once daily proton pump inhibitor. Aliment Pharmacol Ther. 2016;43(8):899-909.
112. Savarino V, Pace F, Scarpignato C, Group ES. Randomised clinical trial: mucosal protection combined with acid suppression in the treatment of non-erosive reflux disease - efficacy of Esoxx, a hyaluronic acid-chondroitin sulphate based bioadhesive formulation. Aliment Pharmacol Ther. 2017;45(5):631-42.
113. Krarup AL, Ny L, Astrand M, Bajor A, Hvid-Jensen F, Hansen MB, et al. Randomised clinical trial: the efficacy of a transient receptor potential vanilloid 1 antagonist AZD1386 in human oesophageal pain. Aliment Pharmacol Ther. 2011;33(10):1113-22.
114. Krarup AL, Ny L, Gunnarsson J, Hvid-Jensen F, Zetterstrand S, Simrén M, et al. Randomized clinical trial: inhibition of the TRPV1 system in patients with nonerosive gastroesophageal reflux disease and a partial response to PPI treatment is not associated with analgesia to esophageal experimental pain. Scand J Gastroenterol. 2013;48(3):274-84.
115. Broekaert D, Fischler B, Sifrim D, Janssens J, Tack J. Influence of citalopram, a selective serotonin reuptake inhibitor, on oesophageal hypersensitivity: a double-blind, placebo-controlled study. Aliment Pharmacol Ther. 2006;23(3):365-70.
116. Peghini PL, Katz PO, Castell DO. Imipramine decreases oesophageal pain perception in human male volunteers. Gut. 1998;42(6):807-13.
117. Weijenborg PW, de Schepper HS, Smout AJ, Bredenoord AJ. Effects of antidepressants in patients with functional esophageal disorders or gastroesophageal reflux disease: a systematic review. Clin Gastroenterol Hepatol. 2015;13(2):251-9.e1.

118. Kandulski A, Wex T, Mönkemüller K, Kuester D, Fry LC, Roessner A, et al. Proteinase-activated receptor-2 in the pathogenesis of gastroesophageal reflux disease. Am J Gastroenterol. 2010;105(9):1934-43.
119. Orlando LA, Orlando RC. Dilated intercellular spaces as a marker of GERD. Curr Gastroenterol Rep. 2009;11(3):190-4.
120. Aziz Q. Visceral hypersensitivity: fact or fiction. Gastroenterology. 2006;131(2):661-4.
121. Thorsell A. Brain neuropeptide Y and corticotropin-releasing hormone in mediating stress and anxiety. Exp Biol Med (Maywood). 2010;235(10):1163-7.
122. Taché Y, Perdue MH. Role of peripheral CRF signalling pathways in stress-related alterations of gut motility and mucosal function. Neurogastroenterol Motil. 2004;16 Suppl 1:137-42.
123. Roman S, Gyawali CP, Savarino E, Yadlapati R, Zerbib F, Wu J, et al. Ambulatory reflux monitoring for diagnosis of gastro-esophageal reflux disease: Update of the Porto consensus and recommendations from an international consensus group. Neurogastroenterol Motil. 2017.
124. Kahrilas PJ, Sifrim D. High-resolution manometry and impedance-pH/manometry: valuable tools in clinical and investigational esophagology. Gastroenterology. 2008;135(3):756-69.
125. Sifrim D, Castell D, Dent J, Kahrilas PJ. Gastro-oesophageal reflux monitoring: review and consensus report on detection and definitions of acid, non-acid, and gas reflux. Gut. 2004;53(7):1024-31.
126. Drewes AM, Schipper KP, Dimcevski G, Petersen P, Andersen OK, Gregersen H, et al. Multimodal assessment of pain in the esophagus: a new experimental model. Am J Physiol Gastrointest Liver Physiol. 2002;283(1):G95-103.
127. Drewes AM, Schipper KP, Dimcevski G, Petersen P, Andersen OK, Gregersen H, et al. Multi-modal induction and assessment of allodynia and hyperalgesia in the human oesophagus. Eur J Pain. 2003;7(6):539-49.
128. Drewes AM, Gregersen H, Arendt-Nielsen L. Experimental pain in gastroenterology: a reappraisal of human studies. Scand J Gastroenterol. 2003;38(11):1115-30.
129. Frøbert O, Arendt-Nielsen L, Bak P, Andersen OK, Funch-Jensen P, Bagger JP. Electric stimulation of the esophageal mucosa. Perception and brain-evoked potentials. Scand J Gastroenterol. 1994;29(9):776-81.
130. Bernstein LM, Fruin RD, Pacini R. Differentiation of esophageal pain from angina pectoris: role of the esophageal acid perfusion test. Medicine (Baltimore). 1962;41:143-62.
131. Watson D, Clark LA, Tellegen A. Development and validation of brief measures of positive and negative affect: the PANAS scales. J Pers Soc Psychol. 1988;54(6):1063-70.
132. Van der Ploeg HDS. Handleiding bij de zelfbeoordelings Vragenlijst (ZBV). Een Nederlandse bewerking van de Spielberger State-Trait Anxiety Inventory (STAI-DY). : Lisse: Swets & Zeitlinger; 1980.
133. Wallon C, Braaf Y, Wolving M, Olaison G, Söderholm JD. Endoscopic biopsies in Ussing chambers evaluated for studies of macromolecular permeability in the human colon. Scand J Gastroenterol. 2005;40(5):586-95.
134. Omari T, Tack J, Rommel N. Impedance as an adjunct to manometric testing to investigate symptoms of dysphagia: What it has failed to do and what it may tell us in the future. United European Gastroenterol J. 2014;2(5):355-66.
135. Kahrilas PJ, Bredenoord AJ, Fox M, Gyawali CP, Roman S, Smout AJ, et al. The Chicago Classification of esophageal motility disorders, v3.0. Neurogastroenterol Motil. 2015;27(2):160-74.
136. Rommel N, Van Oudenhove L, Tack J, Omari TI. Automated impedance manometry analysis as a method to assess esophageal function. Neurogastroenterol Motil. 2014;26(5):636-45.
137. Omari TI, Wauters L, Rommel N, Kritas S, Myers JC. Oesophageal pressure-flow metrics in relation to bolus volume, bolus consistency, and bolus perception. United European Gastroenterol J. 2013;1(4):249-58.
138. Rommel N, Omari TI, Selleslagh M, Kritas S, Cock C, Rosan R, et al. High-resolution manometry combined with impedance measurements discriminates the cause of dysphagia in children. Eur J Pediatr. 2015;174(12):1629-37.

139. Savarino E, Tutuian R, Zentilin P, Dulbecco P, Pohl D, Marabotto E, et al. Characteristics of reflux episodes and symptom association in patients with erosive esophagitis and nonerosive reflux disease: study using combined impedance-pH off therapy. Am J Gastroenterol. 2010;105(5):1053-61.
140. Savarino E, de Bortoli N, De Cassan C, Della Coletta M, Bartolo O, Furnari M, et al. The natural history of gastro-esophageal reflux disease: a comprehensive review. Dis Esophagus. 2017;30(2):1-9.
141. Vaezi MF, Singh S, Richter JE. Role of acid and duodenogastric reflux in esophageal mucosal injury: a review of animal and human studies. Gastroenterology. 1995;108(6):1897-907.
142. Chiba N, De Gara CJ, Wilkinson JM, Hunt RH. Speed of healing and symptom relief in grade II to IV gastroesophageal reflux disease: a meta-analysis. Gastroenterology. 1997;112(6):1798-810.
143. Sandhu DS, Fass R. Current Trends in the Management of Gastroesophageal Reflux Disease. Gut Liver. 2017:10.
144. Fass R. Therapeutic options for refractory gastroesophageal reflux disease. J Gastroenterol Hepatol. 2012;27 Suppl 3:3-7.
145. Boecxstaens V PA, Blondeau K, Oustamanolakis P, Altan E, Boeckxstaens G, Tack J. Refractory GERD patients display increased visceral hypersensitivity for thermal, chemical and mechanical esophageal stimulation. Poster presentation, Digestive Disease Week, Orlando. 2013.
146. Farré R, De Vos R, Geboes K, Verbecke K, Vanden Berghe P, Depoortere I, et al. Critical role of stress in increased oesophageal mucosa permeability and dilated intercellular spaces. Gut. 2007;56(9):1191-7.
147. Naliboff BD, Mayer M, Fass R, Fitzgerald LZ, Chang L, Bolus R, et al. The effect of life stress on symptoms of heartburn. Psychosom Med. 2004;66(3):426-34.
148. Becher A, El-Serag H. Systematic review: the association between symptomatic response to proton pump inhibitors and health-related quality of life in patients with gastro-oesophageal reflux disease. Aliment Pharmacol Ther. 2011;34(6):618-27.
149. Vela MF, Camacho-Lobato L, Srinivasan R, Tutuian R, Katz PO, Castell DO. Simultaneous intraesophageal impedance and pH measurement of acid and nonacid gastroesophageal reflux: effect of omeprazole. Gastroenterology. 2001;120(7):1599-606.
150. Zerbib F, Duriez A, Roman S, Capdepont M, Mion F. Determinants of gastro-oesophageal reflux perception in patients with persistent symptoms despite proton pump inhibitors. Gut. 2008;57(2):156-60.
151. Tsoukali E, Sifrim D. The role of weakly acidic reflux in proton pump inhibitor failure, has dust settled? J Neurogastroenterol Motil. 2010;16(3):258-64.
152. Richter JE, Bradley LA, DeMeester TR, Wu WC. Normal 24-hr ambulatory esophageal pH values. Influence of study center, pH electrode, age, and gender. Dig Dis Sci. 1992;37(6):849-56.
153. Fass R, Sampliner RE, Mackel C, McGee D, Rappaport W. Age- and gender-related differences in 24-hour esophageal pH monitoring of normal subjects. Dig Dis Sci. 1993;38(10):1926-8.
154. Becher A, Dent J. Systematic review: ageing and gastro-oesophageal reflux disease symptoms, oesophageal function and reflux oesophagitis. Aliment Pharmacol Ther. 2011;33(4):442-54.
155. Jung B, Steinbach J, Beaumont C, Mittal RK. Lack of association between esophageal acid sensitivity detected by prolonged pH monitoring and Bernstein testing. Am J Gastroenterol. 2004;99(3):410-5.
156. Prakash C, Clouse RE. Value of extended recording time with wireless pH monitoring in evaluating gastroesophageal reflux disease. Clin Gastroenterol Hepatol. 2005;3(4):329-34.
157. Prakash C, Clouse RE. Wireless pH monitoring in patients with non-cardiac chest pain. Am J Gastroenterol. 2006;101(3):446-52.
158. Weltens N, Schaub N, Van Oudenhove L, Ly HG, Aziz Q, Tack J, et al. Positive and Negative Mood Modulate Esophageal Pain Perception in Health. Gastroenterology. 2013;144(5):S556.
159. Blondeau K, Pauwels A, Boecxstaens V, Caenepeel C, Depeyper S, Holvoet L, et al. Affective Disorders, Somatization and Body Awareness are Associated With Positive Symptom Association During 24 Hour Impedance-pH Monitoring in Patients With Reflux Symptoms. Digestive Disease Week; San Diego, USA: Gastroenterology; 2012. p. S-204.

160. Pardon N. Evaluation of impaired esophageal mucosal integrity in the pathology of refractory gastroesophageal reflux disease. Leuven: KU Leuven; 2017.
161. Pauwels A, Zerbib F, Roman S, Tack J, Farré Ricard. A Low mucosal integrity in a subgroup of healthy subjects increases with proton pump inhibitor treatment. Gastroenterology. 2014;146(5):S861-S.
162. Xie C, Sifrim D, Li Y, Chen M, Xiao Y. Esophageal Baseline Impedance Reflects Mucosal Integrity and Predicts Symptomatic Outcome With Proton Pump Inhibitor Treatment. J Neurogastroenterol Motil. 2017.
163. de Bortoli N, Martinucci I, Savarino E, Tutuian R, Frazzoni M, Piaggi P, et al. Association between baseline impedance values and response proton pump inhibitors in patients with heartburn. Clin Gastroenterol Hepatol. 2015;13(6):1082-8.e1.
164. Kessing BF, Bredenoord AJ, Weijenborg PW, Hemmink GJ, Loots CM, Smout AJ. Esophageal acid exposure decreases intraluminal baseline impedance levels. Am J Gastroenterol. 2011;106(12):2093-7.
165. Cottrell GS, Amadesi S, Schmidlin F, Bunnett N. Protease-activated receptor 2: activation, signalling and function. Biochem Soc Trans. 2003;31(Pt 6):1191-7.
166. Mayer EA. Gut feelings: the emerging biology of gut-brain communication. Nat Rev Neurosci. 2011;12(8):453-66.
167. Grundy D. Signalling the state of the digestive tract. Auton Neurosci. 2006;125(1-2):76-80.
168. Kirkup AJ, Brunsden AM, Grundy D. Receptors and transmission in the brain-gut axis: potential for novel therapies. I. Receptors on visceral afferents. Am J Physiol Gastrointest Liver Physiol. 2001;280(5):G787-94.
169. Aziz Q, Fass R, Gyawali CP, Miwa H, Pandolfino JE, Zerbib F. Functional Esophageal Disorders. Gastroenterology. 2016.
170. Vanner S, Greenwood-Van Meerveld B, Mawe G, Shea-Donohue T, Verdu EF, Wood J, et al. Fundamentals of Neurogastroenterology: Basic Science. Gastroenterology. 2016;S0016-5085(16):00184-0.
171. Ballantyne JC, Sullivan MD. The discovery of endogenous opioid systems: what it has meant for the clinician's understanding of pain and its treatment. Pain. 2017;158(12):2290-300.
172. Wood JD, Galligan JJ. Function of opioids in the enteric nervous system. Neurogastroenterol Motil. 2004;16 Suppl 2:17-28.
173. Vuong C, Van Uum SH, O'Dell LE, Lutfy K, Friedman TC. The effects of opioids and opioid analogs on animal and human endocrine systems. Endocr Rev. 2010;31(1):98-132.
174. Sternini C, Patierno S, Selmer IS, Kirchgessner A. The opioid system in the gastrointestinal tract. Neurogastroenterol Motil. 2004;16 Suppl 2:3-16.
175. Ratuapli SK, Crowell MD, DiBaise JK, Vela MF, Ramirez FC, Burdick GE, et al. Opioid-Induced Esophageal Dysfunction (OIED) in Patients on Chronic Opioids. Am J Gastroenterol. 2015;110(7):979-84.
176. Rattan S, Goyal RK. Identification and localization of opioid receptors in the opossum lower esophageal sphincter. J Pharmacol Exp Ther. 1983;224(2):391-7.
177. Van Oudenhove L, Demyttenaere K, Tack J, Aziz Q. Central nervous system involvement in functional gastrointestinal disorders. Best Pract Res Clin Gastroenterol. 2004;18(4):663-80.
178. Ribeiro SC, Kennedy SE, Smith YR, Stohler CS, Zubieta JK. Interface of physical and emotional stress regulation through the endogenous opioid system and mu-opioid receptors. Prog Neuropsychopharmacol Biol Psychiatry. 2005;29(8):1264-80.
179. LY HG. Brain mechanisms of visceral pain perception and meal volume tolerance in health and functional dyspepsia. Leuven, Belgium: KU Leuven; 2014.
180. Maarrawi J, Peyron R, Mertens P, Costes N, Magnin M, Sindou M, et al. Differential brain opioid receptor availability in central and peripheral neuropathic pain. Pain. 2007;127(1-2):183-94.
181. Harris RE, Clauw DJ, Scott DJ, McLean SA, Gracely RH, Zubieta JK. Decreased central mu-opioid receptor availability in fibromyalgia. J Neurosci. 2007;27(37):10000-6.

182. Staahl C, Christrup LL, Andersen SD, Arendt-Nielsen L, Drewes AM. A comparative study of oxycodone and morphine in a multi-modal, tissue-differentiated experimental pain model. Pain. 2006;123(1-2):28-36.
183. Ly HG, Dupont P, Geeraerts B, Bormans G, Van Laere K, Tack J, et al. Lack of endogenous opioid release during sustained visceral pain: a [11C]carfentanil PET study. Pain. 2013;154(10):2072-7.
184. Geeraerts B, Mimidis K, van Oudenhove L, Vos R, Karamanolis G, Tack J. Role of endogenous opioids in the control of gastric sensorimotor function. Neurogastroenterol Motil. 2008;20(10):1094-102.
185. Janssen P, Pottel H, Vos R, Tack J. Endogenously released opioids mediate meal-induced gastric relaxation via peripheral mu-opioid receptors. Aliment Pharmacol Ther. 2011;33(5):607-14.
186. Arendt-Nielsen L, Olesen AE, Staahl C, Menzaghi F, Kell S, Wong GY, et al. Analgesic efficacy of peripheral kappa-opioid receptor agonist CR665 compared to oxycodone in a multi-modal, multi-tissue experimental human pain model: selective effect on visceral pain. Anesthesiology. 2009;111(3):616-24.
187. Przewłocki R, Przewłocka B. Opioids in chronic pain. Eur J Pharmacol. 2001;429(1-3):79-91.
188. Labuz D, Celik M, Zimmer A, Machelska H. Distinct roles of exogenous opioid agonists and endogenous opioid peptides in the peripheral control of neuropathy-triggered heat pain. Sci Rep. 2016;6:32799.
189. Dubin AE, Patapoutian A. Nociceptors: the sensors of the pain pathway. J Clin Invest. 2010;120(11):3760-72.
190. Kahrilas PJ, Keefer L, Pandolfino JE. Patients with refractory reflux symptoms: What do they have and how should they be managed? Neurogastroenterol Motil. 2015;27(9):1195-201.
191. Michel K, Zeller F, Langer R, Nekarda H, Kruger D, Dover TJ, et al. Serotonin excites neurons in the human submucous plexus via 5-HT3 receptors. Gastroenterology. 2005;128(5):1317-26.
192. Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology. 2007;132(1):397-414.
193. Boecxstaens V. Mechanisms and predictors of symptom perception and therapeutic outcome in refractory gastro-oesophageal reflux disease. Leuven: KU Leuven; 2013.
194. Di Stefano M, Papathanasopoulos A, Blondeau K, Vos R, Boecxstaens V, Farré R, et al. Effect of buspirone, a 5-HT1A receptor agonist, on esophageal motility in healthy volunteers. Dis Esophagus. 2012;25(5):470-6.
195. Hood SD, Bell CJ, Nutt DJ. Acute tryptophan depletion. Part I: rationale and methodology. Aust N Z J Psychiatry. 2005;39(7):558-64.
196. Tack J, Vos R, Janssens J, Salter J, Jauffret S, Vandeplassche G. Influence of tegaserod on proximal gastric tone and on the perception of gastric distension. Aliment Pharmacol Ther. 2003;18(10):1031-7.
197. Kilkens TO, Honig A, van Nieuwenhoven MA, Riedel WJ, Brummer RJ. Acute tryptophan depletion affects brain-gut responses in irritable bowel syndrome patients and controls. Gut. 2004;53(12):1794-800.
198. Geeraerts B, Vandenberghe J, Van Oudenhove L, Gregory LJ, Aziz Q, Dupont P, et al. Influence of experimentally induced anxiety on gastric sensorimotor function in humans. Gastroenterology. 2005;129(5):1437-44.
199. Bell CJ, Hood SD, Nutt DJ. Acute tryptophan depletion. Part II: clinical effects and implications. Aust N Z J Psychiatry. 2005;39(7):565-74.
200. Fernstrom JD. Diet-induced changes in plasma amino acid pattern: effects on the brain uptake of large neutral amino acids, and on brain serotonin synthesis. J Neural Transm Suppl. 1979(15):55-67.
201. Krarup AL, Gunnarsson J, Brun J, Poulakis A, Edebo A, Ringström G, et al. Exploration of the effects of gender and mild esophagitis on esophageal pain thresholds in the normal and sensitized state of asymptomatic young volunteers. Neurogastroenterol Motil. 2013;25(9):766-e580.
202. Nguyen P, Lee SD, Castell DO. Evidence of gender differences in esophageal pain threshold. Am J Gastroenterol. 1995;90(6):901-5.

203. Reddy H, Arendt-Nielsen L, Staahl C, Pedersen J, Funch-Jensen P, Gregersen H, et al. Gender differences in pain and biomechanical responses after acid sensitization of the human esophagus. Dig Dis Sci. 2005;50(11):2050-8.
204. Smith SE, Pihl RO, Young SN, Ervin FR. A test of possible cognitive and environmental influences on the mood lowering effect of tryptophan depletion in normal males. Psychopharmacology (Berl). 1987;91(4):451-7.
205. Ellenbogen MA, Young SN, Dean P, Palmour RM, Benkelfat C. Mood response to acute tryptophan depletion in healthy volunteers: sex differences and temporal stability. Neuropsychopharmacology. 1996;15(5):465-74.
206. Glavin GB, Szabo S. Dopamine in gastrointestinal disease. Dig Dis Sci. 1990;35(9):1153-61.
207. Vaughan CJ, Aherne AM, Lane E, Power O, Carey RM, O'Connell DP. Identification and regional distribution of the dopamine D(1A) receptor in the gastrointestinal tract. Am J Physiol Regul Integr Comp Physiol. 2000;279(2):R599-609.
208. Missale C, Nash SR, Robinson SW, Jaber M, Caron MG. Dopamine receptors: from structure to function. Physiol Rev. 1998;78(1):189-225.
209. Missale G, Missale C, Sigala S, Cestari R, Memo M, Lojacono L, et al. Evidence for the presence of both D-1 and D-2 dopamine receptors in human esophagus. Life Sci. 1990;47(5):447-55.
210. Liu XB, Liu JF. Expression of dopamine receptors in human lower esophageal sphincter. J Gastroenterol Hepatol. 2012;27(5):945-50.
211. Abrahamsson H. Treatment options for patients with severe gastroparesis. Gut. 2007;56(6):877-83.
212. Sigala S, Missale G, Raddino R, Cestari R, Lojacono L, Missale C, et al. Opposing roles for D-1 and D-2 dopamine receptors in the regulation of lower esophageal sphincter motility in the rat. Life Sci. 1994;54(15):1035-45.
213. Valenzuela JE, Dooley CP. Dopamine antagonists in the upper gastrointestinal tract. Scand J Gastroenterol Suppl. 1984;96:127-36.
214. Scarpellini E, Vos R, Blondeau K, Boecxstaens V, Farré R, Gasbarrini A, et al. The effects of itopride on oesophageal motility and lower oesophageal sphincter function in man. Aliment Pharmacol Ther. 2011;33(1):99-105.
215. Kim YS, Kim TH, Choi CS, Shon YW, Kim SW, Seo GS, et al. Effect of itopride, a new prokinetic, in patients with mild GERD: a pilot study. World J Gastroenterol. 2005;11(27):4210-4.
216. Asano T, Tanaka KI, Tada A, Shimamura H, Tanaka R, Maruoka H, et al. Ameliorative effect of chlorpromazine hydrochloride on visceral hypersensitivity in rats: possible involvement of 5-HT2A receptor. Br J Pharmacol. 2017;174(19):3370-81.
217. Yeung PK, Hubbard JW, Korchinski ED, Midha KK. Pharmacokinetics of chlorpromazine and key metabolites. Eur J Clin Pharmacol. 1993;45(6):563-9.
218. Nur S, Adams CE. Chlorpromazine versus reserpine for schizophrenia. Cochrane Database Syst Rev. 2016;4:CD012122.
219. Sanofi. Product information: Largactil. Largactil GLUv11 PIv9 28 Aug 12. 2012.
220. Adams CE, Rathbone J, Thornley B, Clarke M, Borrill J, Wahlbeck K, et al. Chlorpromazine for schizophrenia: a Cochrane systematic review of 50 years of randomised controlled trials. BMC Med. 2005;3:15.
221. Holtmann G, Talley NJ, Liebregts T, Adam B, Parow C. A placebo-controlled trial of itopride in functional dyspepsia. N Engl J Med. 2006;354(8):832-40.
222. Meltzer HY. Update on typical and atypical antipsychotic drugs. Annu Rev Med. 2013;64:393-406.
223. Horacek J, Bubenikova-Valesova V, Kopecek M, Palenicek T, Dockery C, Mohr P, et al. Mechanism of action of atypical antipsychotic drugs and the neurobiology of schizophrenia. CNS Drugs. 2006;20(5):389-409.
224. Bradley LA, Richter JE, Pulliam TJ, Haile JM, Scarinci IC, Schan CA, et al. The relationship between stress and symptoms of gastroesophageal reflux: the influence of psychological factors. Am J Gastroenterol. 1993;88(1):11-9.

225. Dent J, El-Serag HB, Wallander MA, Johansson S. Epidemiology of gastro-oesophageal reflux disease: a systematic review. Gut. 2005;54(5):710-7.
226. Rubin J, Nagler R, Spiro H, Pilot M. Measuring the effect of emotions on esophageal motility. Psychosom Med. 1962;24:170-6.
227. Johnston BT, McFarland RJ, Collins JS, Love AH. Effect of acute stress on oesophageal motility in patients with gastro-oesophageal reflux disease. Gut. 1996;38(4):492-7.
228. Ayres RC, Robertson DA, Naylor K, Smith CL. Stress and oesophageal motility in normal subjects and patients with irritable bowel syndrome. Gut. 1989;30(11):1540-3.
229. Stacher G, Schmierer G, Landgraf M. Tertiary esophageal contractions evoked by acoustical stimuli. Gastroenterology. 1979;77(1):49-54.
230. Taché Y, Martinez V, Wang L, Million M. CRF1 receptor signaling pathways are involved in stress-related alterations of colonic function and viscerosensitivity: implications for irritable bowel syndrome. Br J Pharmacol. 2004;141(8):1321-30.
231. Vanuytsel T, van Wanrooy S, Vanheel H, Vanormelingen C, Verschueren S, Houben E, et al. Psychological stress and corticotropin-releasing hormone increase intestinal permeability in humans by a mast cell-dependent mechanism. Gut. 2014;63(8):1293-9.
232. Fukudo S, Nomura T, Hongo M. Impact of corticotropin-releasing hormone on gastrointestinal motility and adrenocorticotropic hormone in normal controls and patients with irritable bowel syndrome. Gut. 1998;42(6):845-9.
233. Suda T, Tomori N, Yajima F, Sumitomo T, Nakagami Y, Ushiyama T, et al. Immunoreactive corticotropin-releasing factor in human plasma. J Clin Invest. 1985;76(5):2026-9.
234. Cohen J. Statistical power analysis for the behavioral sciences. 2 ed. Hillsdale, NJ: Lawrence Earlbaum Associates; 1988.
235. Anderson KO, Dalton CB, Bradley LA, Richter JE. Stress induces alteration of esophageal pressures in healthy volunteers and non-cardiac chest pain patients. Dig Dis Sci. 1989;34(1):83-91.
236. Nozu T, Kudaira M. Corticotropin-releasing factor induces rectal hypersensitivity after repetitive painful rectal distention in healthy humans. J Gastroenterol. 2006;41(8):740-4.
237. Sagami Y, Shimada Y, Tayama J, Nomura T, Satake M, Endo Y, et al. Effect of a corticotropin releasing hormone receptor antagonist on colonic sensory and motor function in patients with irritable bowel syndrome. Gut. 2004;53(7):958-64.
238. Larauche M, Gourcerol G, Wang L, Pambukchian K, Brunnhuber S, Adelson DW, et al. Cortagine, a CRF1 agonist, induces stresslike alterations of colonic function and visceral hypersensitivity in rodents primarily through peripheral pathways. Am J Physiol Gastrointest Liver Physiol. 2009;297(1):G215-27.
239. Mayer EA. The neurobiology of stress and gastrointestinal disease. Gut. 2000;47(6):861-9.
240. Larauche M. Novel insights in the role of peripheral corticotropin-releasing factor and mast cells in stress-induced visceral hypersensitivity. Neurogastroenterol Motil. 2012;24(3):201-5.
241. Kastin AJ, Pan W, Maness LM, Banks WA. Peptides crossing the blood-brain barrier: some unusual observations. Brain Res. 1999;848(1-2):96-100.
242. Zheng PY, Feng BS, Oluwole C, Struiksma S, Chen X, Li P, et al. Psychological stress induces eosinophils to produce corticotrophin releasing hormone in the intestine. Gut. 2009;58(11):1473-9.
243. Theoharides TC, Donelan JM, Papadopoulou N, Cao J, Kempuraj D, Conti P. Mast cells as targets of corticotropin-releasing factor and related peptides. Trends Pharmacol Sci. 2004;25(11):563-8.
244. Wallon C, Yang PC, Keita AV, Ericson AC, McKay DM, Sherman PM, et al. Corticotropin-releasing hormone (CRH) regulates macromolecular permeability via mast cells in normal human colonic biopsies in vitro. Gut. 2008;57(1):50-8.
245. Taché Y, Million M. Role of Corticotropin-releasing Factor Signaling in Stress-related Alterations of Colonic Motility and Hyperalgesia. J Neurogastroenterol Motil. 2015;21(1):8-24.
246. Binder EB, Nemeroff CB. The CRF system, stress, depression and anxiety-insights from human genetic studies. Mol Psychiatry. 2010;15(6):574-88.
247. Kanazawa M, Hongo M, Fukudo S. Visceral hypersensitivity in irritable bowel syndrome. J Gastroenterol Hepatol. 2011;26 Suppl 3:119-21.

248. Reddy H, Staahl C, Arendt-Nielsen L, Gregersen H, Drewes AM, Funch-Jensen P. Sensory and biomechanical properties of the esophagus in non-erosive reflux disease. Scand J Gastroenterol. 2007;42(4):432-40.
249. Holloway RH. Combined impedance-manometry for the evaluation of esophageal disorders. Curr Opin Gastroenterol. 2014;30(4):422-7.
250. Pandolfino JE, Bulsiewicz WJ. Evaluation of esophageal motor disorders in the era of high-resolution manometry and intraluminal impedance. Curr Gastroenterol Rep. 2009;11(3):182-9.
251. Herregods TV, Roman S, Kahrilas PJ, Smout AJ, Bredenoord AJ. Normative values in esophageal high-resolution manometry. Neurogastroenterol Motil. 2015;27(2):175-87.
252. Mittal RK, Stewart WR, Ramahi M, Chen J, Tisdelle D. The effects of psychological stress on the esophagogastric junction pressure and swallow-induced relaxation. Gastroenterology. 1994;106(6):1477-84.
253. Cook IJ, Dent J, Collins SM. Upper esophageal sphincter tone and reactivity to stress in patients with a history of globus sensation. Dig Dis Sci. 1989;34(5):672-6.
254. Rodrigo J, Pedrosa JA, Alvarez FJ, Bentura ML, Uttenthal O, Martinez-Murillo R, et al. Presence of calcitonin gene-related peptide in intraepithelial nerve fibers and motor end-plates of the cat esophagus: a light and electron microscopic study. J Auton Nerv Syst. 1994;49(1):21-31.
255. Guarino MP, Cheng L, Ma J, Harnett K, Biancani P, Altomare A, et al. Increased TRPV1 gene expression in esophageal mucosa of patients with non-erosive and erosive reflux disease. Neurogastroenterol Motil. 2010;22(7):746-51, e219.
256. Farmer AD, Aziz Q. Gut pain & visceral hypersensitivity. Br J Pain. 2013;7(1):39-47.
257. Farmer AD, Aziz Q. Visceral pain hypersensitivity in functional gastrointestinal disorders. Br Med Bull. 2009;91:123-36.
258. Viazis N, Keyoglou A, Kanellopoulos AK, Karamanolis G, Vlachogiannakos J, Triantafyllou K, et al. Selective serotonin reuptake inhibitors for the treatment of hypersensitive esophagus: a randomized, double-blind, placebo-controlled study. Am J Gastroenterol. 2012;107(11):1662-7.
259. Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, et al. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacological reviews. 2002;54(2):161-202.
260. Wilson RI, Nicoll RA. Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. Nature. 2001;410(6828):588-92.
261. Sanson M, Bueno L, Fioramonti J. Involvement of cannabinoid receptors in inflammatory hypersensitivity to colonic distension in rats. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2006;18(10):949-56.
262. Kikuchi A, Ohashi K, Sugie Y, Sugimoto H, Omura H. Pharmacological evaluation of a novel cannabinoid 2 (CB2) ligand, PF-03550096, in vitro and in vivo by using a rat model of visceral hypersensitivity. Journal of pharmacological sciences. 2008;106(2):219-24.
263. Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, et al. Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. Nature medicine. 2007;13(1):35-7.
264. Scarpellini E, Blondeau K, Boecxstaens V, Vos R, Gasbarrini A, Farre R, et al. Effect of rimonabant on oesophageal motor function in man. Alimentary pharmacology & therapeutics. 2011;33(6):730-7.

CHAPTER 9

SUMMARY / SAMENVATTING

9 Summary / Samenvatting

9.1 Summary

Gastro-esophageal reflux (GER) is the retrograde flow of gastric contents into the esophagus. When GER is causing troublesome symptoms, such as heartburn or regurgitation, or lesions, it is referred to as gastro-esophageal reflux disease (GERD). GERD is a frequent condition affecting about 10 to 30% of the adult Western population. It may present with a broad spectrum of symptoms, divided into typical or esophageal manifestations of which heartburn and regurgitation are most important and a variety of atypical, extra-esophageal symptoms, such as chronic cough, wheezing and hoarseness. Acid suppressive therapy, especially treatment with PPIs, is the first treatment option in patients with GERD symptoms. Unfortunately, 10 to 40% of patients continue to experience reflux symptoms despite optimized PPI therapy and are said to have refractory GERD (rGERD). The pathophysiology of rGERD symptoms remains incompletely understood. The overall objective of this PhD project was to investigate why rGERD patients continue to experience symptoms while on a double dose of PPI. Therefore, we have investigated which underlying mechanisms could explain ongoing symptoms of GERD. In this project, we focused on the involvement of esophageal hypersensitivity and if an impaired esophageal epithelial integrity could explain sensitivity changes in rGERD patients. Furthermore, we studied the role of three different anti-nociceptive pathways in the modulation of esophageal sensitivity and the effect of psychosocial factors such as stress on esophageal pain perception in health.

After characterization of reflux parameters in rGERD on PPI therapy we demonstrated that ongoing non-acid reflux, volume exposure and the number of reflux events with a high proximal extent seem to be involved in symptom generation in rGERD. Esophageal sensitivity was assessed using a multimodal stimulation protocol. Four stimulation modalities were applied to evaluate esophageal sensitivity: thermal, mechanical, electrical, and chemical stimulation. Multimodal esophageal stimulation revealed that rGERD patients have an increased esophageal sensitivity to electrical and chemical stimulation indicating that altered esophageal sensitivity is involved in symptom perception in these patients. In rGERD patients on a double dose of PPIs, impaired esophageal epithelial integrity measured *in vitro* by Ussing chamber experiments and *in vivo* by evaluation of impedance baseline values did not explain this increased sensitivity since no differences in esophageal integrity were present when our

rGERD cohort was compared to HV. We demonstrated that feelings of anxiety and negative affect are more present in rGERD patients compared to HV and are likely to play a role in an altered perception of GERD symptoms. To clarify if failure of descending anti-nociceptive pathways was involved in esophageal sensitivity changes in rGERD, we investigated the effect of blocking endogenous opioids, the serotoninergic system and the dopaminergic system on esophageal sensitivity in healthy volunteers by using the multimodal stimulation protocol. The serotonin system seems to be a useful target for future therapies in patients with reflux hypersensitivity and functional heartburn since low levels of serotonin are related to increased acid sensitivity. Peripheral and central antagonism of μ-opioid receptors and blocking the dopamine 2 receptor did not alter esophageal sensitivity to multimodal stimulation in healthy volunteers. Finally, since psychosocial stressors have been demonstrated to be important in modulation of esophageal sensation, we investigated the effect of peripheral administration of the stress hormone CRH on esophageal sensitivity and motility in health. We demonstrated that IV CRH administration increased esophageal sensitivity to mechanical distention in health. Furthermore, we observed an increase in esophageal contractility and tone and a decrease in LES relaxation. As expected peripheral CRH administration increased cortisol levels. The changes in esophageal contractile properties may underlie the increased sensitivity to balloon distention after CRH.

In conclusion, rGERD is a very complex spectrum disease with a broad range of pathologic consequences. More research is needed to enhance our knowledge concerning PPI failure in patients with refractory symptoms of GERD.

9.2 Samenvatting

Gastro-oesofageale reflux (GOR) is de retrograde terugvloei (regurgitatie) van maaginhoud in de slokdarm. Wanneer reflux van maaginhoud zorgwekkende symptomen veroorzaakt, zoals brandend maagzuur of regurgitatie, of letsels in het slokdarmlichaam, wordt dit aangeduid als gastro-oesofageale refluxziekte (GORZ). GORZ is een frequente aandoening, ongeveer 10 tot 30% van de volwassen Westerse bevolking lijdt aan deze ziekte. GORZ gaat gepaard met een breed spectrum van symptomen die verdeeld kunnen worden in typische, ofwel oesofageale klachten waarvan zuurbranden en regurgitatie de belangrijkste zijn. Daarenboven is er ook een grote verscheidenheid aan atypische, extra-oesofageale symptomen, zoals chronische hoest, globus en heesheid. Therapie met zuurrremmende medicatie, zoals behandeling met proton pomp inhibitoren (PPI's), is de eerstelijns behandeling bij patiënten met GORZ symptomen. Helaas ondervinden 10 tot 40% van de patiënten nog steeds refluxsymptomen ondanks een geoptimaliseerde PPI-therapie. In dit geval spreekt men van refractaire GORZ (rGORZ). De pathofysiologie van rGORZ symptomen is nog niet volledig opgehelderd. De voornaamste doelstelling van dit doctoraatsonderzoek was om verder te bestuderen waarom patiënten met refractaire GORZ nog steeds symptomen ondervinden, zelfs wanneer ze behandeld worden met een dubbele dosis PPI. Daarom hebben we onderzocht welke onderliggende mechanismen de aanhoudende symptomen van GORZ kunnen verklaren. In dit project lag de nadruk op de invloed van slokdarmgevoeligheid (hypersensitiviteit) op symptoomontwikkeling. Verder bestudeerden we of een aangetaste slokdarmintegriteit de veranderingen in slokdarmsensitiviteit bij rGORZ patiënten zou kunnen verklaren. Daarnaast hebben we de rol van drie verschillende anti-nociceptieve neurotransmittersystemen bestudeerd in de modulatie van slokdarmgevoeligheid alsook het effect van psychosociale factoren, zoals bijvoorbeeld stress, op pijnperceptie in de slokdarm in gezonde proefpersonen.

Na het bepalen van refluxparameters in rGORZ patienten onder PPI-therapie, bleek dat niet-zure reflux episodes, volumeblootstelling en het aantal reflux episodes met een hoge proximale extensie betrokken lijken te zijn bij het veroorzaken van symptomen bij patiënten met rGORZ. De gevoeligheid van de slokdarm werd beoordeeld aan de hand van een multimodale stimulatietest. Vier verschillende stimulatiemodaliteiten werden gebruikt om slokdarmsensitiviteit te evalueren: thermische, mechanische, elektrische en chemische stimulatie. Uit deze multimodale slokdarmstimulatie testen bleek dat rGORZ patiënten een

verhoogde gevoeligheid hebben voor elektrische en chemische stimulatie, wat aangeeft dat veranderde slokdarmgevoeligheid een rol speelt in het genereren van symptomen in deze populatie en dat dit betrokken is bij de perceptie van GORZ symptomen. In onze patiëntengroep was deze verhoogde slokdarmgevoeligheid niet gerelateerd aan een aangetaste slokdarmintegriteit aangezien er geen verschillen in slokdarmintegriteit werden vastgesteld wanneer de rGORZ patiënten werden vergeleken met gezonde proefpersonen. Slokdarmintegriteit werd *in vitro* bestudeerd door middel van de 'Ussing-chamber' techniek en in *vivo* door middel van een evaluatie van basale impedantie-waarden. Voorts hebben we aangetoond dat gevoelens van angst en negatieve emoties meer aanwezig zijn bij rGORZ patiënten ten opzichte van gezonde proefpersonen en dat deze gevoelens waarschijnlijk een rol spelen bij een veranderde perceptie van GORZ symptomen. Om verder te verduidelijken of het falen van anti-nociceptieve neurotransmittersystemen betrokken was bij veranderingen in slokdarmsensitiviteit in rGORZ patiënten, onderzochten we het effect van het blokkeren van endogene opioïden, het serotoninesysteem en het dopaminesysteem op slokdarmsensitiviteit bij gezonde proefpersonen door gebruik te maken van de multimodale stimulatietest. Het serotoninesysteem lijkt een nuttig doelwit te zijn voor toekomstige therapiëen bij patiënten met refluxhypersensitiviteit en bij patiënten met functioneel zuurbranden, aangezien een verlaagde concentratie van serotonine verband houdt met een verhoogde zuurgevoeligheid. Antagonisme van de μ-opioïde receptoren en het blokkeren van de dopamine 2 receptor had geen invloed op slokdarmsensitiviteit bij gezonde proefpersonen. Ten slotte, aangezien het aangetoond is dat psychosociale factoren belangrijk zijn bij modulatie van slokdarmsensatie, hebben we het effect van perifere toediening van het stresshormoon CRH op slokdarmgevoeligheid en -motiliteit in gezonde proefpersonen onderzocht. We hebben aangetoond dat intraveneuze CRH toediening slokdarmsensitiviteit voor mechanische stimulatie verhoogt. Bovendien zagen we een toename in slokdarmcontractiliteit en slokdarmtonus en een afname in de relaxatie van de onderste slokdarmsfincter. Zoals verwacht verhoogde perifere CRH toediening de concentraties van cortisol in het lichaam. De veranderingen in de contractiele eigenschappen van de slokdarm kunnen de verhoogde gevoeligheid voor mechanische stimulatie na CRH verklaren. Samenvattend kunnen we stellen dat rGORZ een zeer complex spectrum van aandoeningen omvat met een breed scala aan pathologische gevolgen. Meer onderzoek is nodig om onze kennis over PPI-falen bij patiënten met refractaire symptomen van GORZ te verbeteren.

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Chapter 1: Introduction

The introduction was written by Charlotte Broers and thoroughly revised by Ans Pauwels and Jan Tack.

Chapter 2: Research objectives

The research objectives of this PhD project were conceived by Jan Tack, Ans Pauwels and Charlotte Broers.

Chapter 3: Materials and Methods

The methods used in this project were described by Charlotte Broers. Ans Pauwels and Jan Tack critically revised this chapter.

Chapter 4: Alterations in esophageal sensitivity and epithelial integrity in refractory GERD

Jan Tack, Ans Pauwels, and Charlotte Broers conceived and designed the study protocol described in this chapter. Jan Tack and Ans Pauwels acquired funding and provided technical services and materials. Jan Tack, Ans Pauwels and Charlotte Broers recruited rGERD patients at the consultation of the outpatient clinic. Additionally, healthy controls were recruited by Charlotte Broers. Charlotte Broers and Ans Pauwels performed experiments and analyzed data. All authors discussed and interpreted results of experiments. Charlotte Broers wrote this chapter and created figures and tables. The chapter was thoroughly revised by Ans Pauwels and Jan Tack.

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Chapter 5: Failure of anti-nociceptive pathways

Jan Tack, Ans Pauwels and Charlotte Broers conceived and designed all the study protocols described in this chapter. Jan Tack and Ans Pauwels acquired funding and provided technical services and

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Chapter 6: Alteration in esophageal pain perception due to stress

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Jan Tack, Ans Pauwels, Charlotte Broers and Chloé Melchior conceived and designed the study protocol. Jan Tack and Ans Pauwels acquired funding and provided technical services and materials. Charlotte Broers and Chloé Melchior recruited participants. Charlotte Broers, Chloé Melchior, Ans Pauwels and Brecht Van Houtte performed experiments. Charlotte Broers, Chloé Melchior analyzed data. Charlotte Broers took the lead in writing the manuscript and prepared tables and figures with support from Ans Pauwels and Chloé Melchior. All authors critically revised and edited the manuscript. Charlotte Broers, Ans Pauwels and Jan Tack finalized the manuscript.

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Chapter 7: General discussion and future prospects

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Summary/Samenvatting

The summary of this project was written by Charlotte Broers and thoroughly revised by Ans Pauwels and Jan Tack.

Conflict of interest

The authors have no conflicts of interest to declare.

Curriculum vitae

Charlotte Broers was born in Genk (Belgium) on May 10th, 1990. After finishing secondary school (Latin-Mathematics) at the Sint-Jan Berchmanscollege in Genk in 2008, she started her training in Biomedical Sciences at the University of Hasselt (Belgium). She obtained her bachelor's degree in 2011 and her master's degree in 2013. Subsequently, she started her PhD training at the Translational Research Center for Gastrointestinal Disorders (TARGID), KU Leuven, under the supervision of Prof. Jan Tack and Dr. Ans Pauwels in October 2013. Her scientific work has contributed to several abstracts and peer-reviewed publications.

List of Publications

Articles in internationally reviewed scientific journals

Broers C, Boecxstaens V, Deloose E, Tack J, Pauwels A. The multimodal esophageal stimulation paradigm: optimal order of stimulation modalities and reproducibility. Submitted to United European Gastroenterology Journal, *major revision*.

Broers C, Tack J, Pauwels A. Review article: Gastro-oesophageal reflux disease in asthma and chronic obstructive pulmonary disease. Aliment Pharmacol Ther. 2018;47:176–191

Pauwels A, **Broers C**, Van Houtte B, Rommel N, Vanuytsel T, Tack J. A randomized double-blind, placebo-controlled, cross-over study using baclofen in the treatment of rumination syndrome. Am J Gastroenterol. 2017 Dec 5, [Epub ahead of print].

Pauwels A, **Broers C**, Cocca S, Vanuytsel T, Pardon N, Roman S, Zerbib F, Tack J, Farré R. A reduced esophageal epithelial integrity in a subgroup of healthy subjects increases with proton pump inhibitor therapy. United European Gastroenterology Journal, December 2017, *in press*.

Scheerens C, Omari T, **Broers C**, Tack J, Rommel N, Esophageal pressure-flow metrics in relation to bolus consistency and bolus perception in patients with esophageal dysphagia: Observations from a large clinical series. Submitted to Journal of Gastroenterology 2017, *minor revision*.

Broers C, Melchior C, Van Oudenhove L, Vanuytsel T, Van Houtte B, Scheerens C, Rommel N, Tack J, Pauwels A. The effect of intravenous corticotrophin-releasing hormone administration on esophageal sensitivity and motility in health. Am J Physiol Gastrointest Liver Physiol. 2017, 312(5):G526-G534.

Pauwels A, Boecxstaens V, **Broers C**, Tack JF. Severely impaired gastric accomodation is a hallmark of post-Nissen functional dyspepsia symptoms. Neurogastroenterol Motil. 2017,29(8).

Scarpellini E, Boecxstaens V, **Broers C**, Vos R, Pauwels A, Tack J. Effect of baclofen on gastric acid pocket in subjects with gastroesophageal reflux disease symptoms. Diseases of the Esophagus, 2016, 29(8): 1054-1063.

Laermans J, **Broers C**, Beckers K, Vancleef L, Steensels S, Thijs T, Tack J, Depoortere I. Shifting the circadian rhythm of feeding in mice induces gastrointestinal, metabolic and immune alterations which are influenced by ghrelin and the core clock gene bmal1. PLoS One. 2014 Oct 16;9(10):e110176.

Conference abstracts

Broers C, Van Houtte B, Vermeersch P, Peersman N, Tack J, Pauwels A. Acute tryptophan depletion increases esophageal sensitivity to acid perfusion in health. Abstract accepted for poster presentation at the European Society of Neurogastroenterology and motility (ESNM), Cork, Ireland, August 2017.

Broers C, Van Houtte B, Vermeersch P, Peersman N, Tack J, Pauwels A. Influence of acute tryptophan depletion on esophageal sensitivity and visceral pain perception in health. Abstract accepted for oral presentation at the Belgian Week of Gastroenterology (BWGE), Antwerp, Belgium and poster presentation at the Digestive Disease Week (DDW), Chicago, USA, May 2017.

Pauwels A, Boecxstaens V, **Broers C**, Iven J, Zhao D, Vanuytsel T, Tack J. A double-blind, placebo-controlled trial with baclofen for the treatment of refractory gastro-esophageal reflux disease. Abstract

accepted for oral presentation at the Belgian Week of Gastroenterology (BWGE), Antwerp, Belgium and the Digestive Disease Week (DDW), Chicago, USA, May 2017.

Broers C, Pardon N, Van Houtte B, Vanheel H, Vanuytsel T, Tack J, Pauwels A, Farré R. Impaired mucosal integrity is present in some refractory GORD patients: the relevance of the refluxate. Abstract accepted for poster presentation (Poster in the spotlight session) at the United European Gastroenterology Week (UEGW), Vienna, Austria, October 2016.

Pauwels A, **Broers C**, Van Houtte B, Rommel N, Vanuytsel T, Tack J. Baclofen treatment for rumination syndrome: a double-blind, placebo-controlled, cross-over study. Abstract accepted for oral presentation at the United European Gastroenterology Week (UEGW), Vienna, Austria, October 2016.

Van Houtte B, **Broers C**, Vanuytsel T, Rommel N, Tack J, Pauwels A. The magnitude of gastric accommodation determines the number of transient lower esophageal sphincter relaxations in health. Abstract accepted for poster presentation at the United European Gastroenterology Week (UEGW), Vienna, Austria, October 2016.

Pauwels A, **Broers C**, Van Houtte B, Rommel N, Vanuytsel T, Tack J. Baclofen treatment for rumination syndrome: a double-blind, placebo-controlled, cross-over study. Abstract accepted for oral presentation at 2nd Federation of Neurogastroenterology and Motility Meeting. San Francisco, USA, August 2016.

Van Houtte B, **Broers C**, Vanuytsel T, Rommel N, Tack J, Pauwels A. The magnitude of gastric accommodation determines the number of transient lower esophageal sphincter relaxations in health. Abstract accepted for poster presentation at 2nd Federation of Neurogastroenterology and Motility Meeting. San Francisco, USA, August 2016.

Scheerens C, Omari T, **Broers C**, Tack J, Rommel N. Pressure flow analysis as a method to assess esophageal function. Abstract accepted for poster presentation at 2nd Federation of Neurogastroenterology and Motility Meeting. San Francisco, USA, August 2016.

Broers C, Van Houtte B, Scheerens S, Tack J, Pauwels A. CRH administration sensitizes the esophagus to balloon distention due to an increase in esophageal contractile amplitude. Abstract accepted for oral presentation at the Digestive Disease Week (DDW), Washington, USA, May 2015.

Melchior C, **Broers C**, Vanuytsel T, Van Oudenhove L, Tack J, Pauwels A, CRH-induced stress increases esophageal sensitivity in health. Abstract accepted for oral presentation at the United European Gastroenterology Week (UEGW), Vienna, Austria, October 2014.

Broers C, Pauwels A, Melchior C, Boecxstaens V, Tack J. Esophageal sensitivity and visceral pain perception in health is not modulated by endogenous opioid release. Abstract accepted for oral presentation at the International Society for Diseases of the Esophagus (ISDE), Vancouver, Canada, September 2014.

Laermans J, **Broers C**, Thijs T, Tack J, Depoortere I, The clock gene Bmal1 regulates ghrelin levels and induces inflammation during restricted feeding. Abstract accepted for oral presentation at the annual meeting of the society for the study of ingestive behavior (SSIB), New Orleans, USA, 2013.