Translational studies in gastroesophageal reflux disease

D. R. de Vries

Translational studies in gastroesophageal reflux disease

D.R. de Vries

ISBN 978-90-9024040-4
Printed by Gildeprint drukkerijen Enschede
Cover: Grootzus (Francien Verhulst, www.grootzus.nl)

Publication of this thesis was supported by the department of Gastroenterology and Hepatology at UMCU, Abbott, Janssen-Cilag, Nycomed, Ferring, Astra Zeneca, Pentax, Boehringer-Ingelheim

Translational studies in gastroesophageal reflux disease

Translationele onderzoeken bij gastro-oesofageale refluxziekte

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 12 maart 2009 des middags te 12.45 uur

door

Durk Rimmer de Vries

geboren op 27 april 1978 te Gouda

Promotoren	Prof.dr. M. Samsom Prof.dr. A.J.P.M. Smout
Co-promotoren	Dr. J.J.M. ter Linde Dr. M.A. van Herwaarden

CONTENTS

Chapter 1.

Introduction

INTRODUCTION

Definition and Epidemiology

Gastroesophageal reflux is a process in which contents of the stomach enter the esophagus through the lower esophageal sphincter (LES). In 2006, the international Montreal consensus group defined GERD as a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications [1]. The incidence and prevalence of GERD are rising, it is estimated to affect 10 – 20% of the inhabitants of Western countries and 5% of Asians [2].

GERD is characterized by three classic symptoms: heartburn, non-cardiac retrosternal pain and regurgitation. Non-specific symptoms are manifold in nature and include dysphagia, odynophagia, globus sensation, chronic unexplained cough, hoarseness, asthma attacks, halitosis and dental erosion. Excess reflux can cause damage to the esophageal lining, ranging from small erosions to more severe damage like circumferential esophagitis, esophageal ulcers and strictures, and intestinal metaplasia (Barrett's esophagus) with varying degrees of dysplasia. This goes to show that GERD is not an innocent condition; it has been widely recognized that Barrett's esophagus is a precursor lesion of esophageal adenocarcinoma, a condition with a rising incidence and poor prognosis [2]. Recently, large cross-sectional surveys showed that symptoms and esophageal damage occur simultaneously in a majority of cases, but many symptomatic subjects did not have damage to the esophageal lining while, on the other hand, many subjects with damage caused by reflux did not report any symptoms [3, 4]. GERD overlaps with other gastrointestinal symptom complexes, foremost functional dyspepsia (FD) and irritable bowel syndrome (IBS) [5]. Symptoms of GERD are perceived as bothersome in a majority of patients and decrease health-related quality of life (HRQoL) [6]. Chapter 2 describes the results of a study of the impact of concomitant FD and IBS on HRQoL in GERD patients.

Pathophysiology

Upon reflux of gastric contents the esophageal lining is subjected to several caustic agents: acid, pepsin and bile. To a certain level the esophageal lining can deal with this through innate defense mechanisms [7], and perception of acid reflux is barred by sensory thresholds, however, these mechanisms are not without limits. When gastroesophageal reflux exceeds the amount that is tolerated at any given moment, problems occur that are collectively termed gastroesophageal reflux *disease*, or GERD.

Pathophysiology of increased esophageal acid exposure

Conditions favoring gastroesophageal reflux include hiatus hernia, transient spatial separation of LES and crural diaphragm and a hypotensive LES. In addition, impaired esophageal motility resulting in inadequate clearance of refluxate [8]. The most important mechanism causing gastroesophageal reflux in patients as well as healthy volunteers is the transient LES relaxation (TLESR). In GERD patients a higher percentage of TLESRs is accompanied by reflux [8]. Presence of a hiatus hernia is associated with increased esophageal acid exposure, esophagitis and symptoms [9, 10]. In patients with a hiatus hernia, in addition to reflux during TLESRs, excess reflux occurs through malfunction of the gastroesophageal barrier during low LES pressure, swallow-associated normal LES relaxations, deep inspiration, and straining [11]. Studies using high-resolution manometry of the esophagogastric junction have shown that separation of the LES and the crural diaphragm favor reflux [12].

Evidence of the association with the rising prevalence of GERD and the obesity epidemic is growing. Obesity is associated with GERD symptoms as well as with hiatal hernia and esophagitis [13, 14]. In obese patients, TLESRs occur more frequently [15]. Intragastric pressure and esophageal acid exposure have been reported to be increased in obese patients, and Barrett's esophagus is more prevalent [16-18].

Pathophysiology of reflux perception

As shown in the aforementioned cross-sectional population surveys, perception of gastroesophageal reflux varies tremendously among subjects[3, 4]. It has been shown that a greater proximal extent of the refluxate [19], as well as the presence of gas in the refluxate [20] favor perception of reflux episodes.

Visceral hypersensitivity plays an important role in the generation of symptoms by gastroesophageal reflux [21-23]. Acidic gastroesophageal reflux leaves the esophagus more sensitive to acid for some time after the reflux has occurred [19]. Furthermore, evidence has been provided that central sensitization plays a role in the perception of acid by GERD patients [24].

The duodenum has its own separate effect on the perception of gastroesophageal reflux. For instance, infusion of lipids into the duodenum made subjects, upon infusion of acid into the esophagus, perceive heartburn more intensely [25].

Diagnostic approach

Typical GERD symptoms without any alarm symptoms like dysphagia or weight loss are usually treated by general practitioners. The differential diagnosis of GERD is not very long, but important to address in the clinical work-up. When alarm symptoms are present or when treatment does not provide relief patients can be referred for endoscopy, foremost to rule out esophageal cancer, but hiatus hernia, esophagitis and Barrett's esophagus can be diagnosed during endoscopy as well. Achalasia, nutcracker esophagus and diffuse esophageal spasms can cause non-cardiac chest pain and/or heartburn as well, furthermore esophageal stasis of ingested substances in achalasia can cause regurgitation. An esophageal manometry study is therefore essential in the diagnostic work-up in a referral center. Aside from diagnosing the aforementioned disorders, the location, relaxation and resting pressure of the LES can be assessed. Low LES resting pressure favors gastroesophageal reflux [26]. The esophageal peristalsis and the amplitude of the contractions are important in clearance of refluxate, failure of esophageal peristaltic motility is one of the factors contributing to GERD[27]. Intraesophageal and intragastric pressures are recorded as well, these parameters and their relations with other GERD parameters as well as with body mass index are explored in chapter 3.

In research settings and tertiary referral centers, the most widely used diagnostic tool for GERD is 24-hour intra-esophageal pH-monitoring with symptom association analysis. With this technique, the percentage of time with pH below 4, measured at a fixed distance proximal to the LES which has been manometrically located, can be assessed while the patient records meal times and recumbent periods in a diary. The recording device usually incorporates an event button the patient can push when a symptom is perceived; afterwards the patient describes this symptom in the diary. The combination of pH-tracings and event markers enables the calculation of the symptom index (SI) and symptom association probability (SAP) [28, 29]. These scores provide information about the attributability to acid reflux of the symptoms the patient experiences. Recently, the advent of combined 24-hour pH-impedance measurement has enabled researchers to provide insight in the role of proximal extent of the refluxate and the role of weakly acidic and gas reflux. 24-hour impedance monitoring stands on the brink of becoming a validated clinical application, it did not play a role yet in the selection of patients in the current thesis.

When the esophageal manometry and pH-metry have not cleared up the reason for the symptoms a patient experiences, and a cardiac cause has been ruled out, the diagnosis functional heartburn can be made. According to the Rome-III criteria, this diagnosis can be made only when symptoms do not improve after a proton pump inhibitor (PPI) has been

administered [30], however the additional value of using a PPI prescription as a diagnostic tool is controversial [31, 32].

Treatment

The only known drug that reduces TLESR frequency is baclofen, which has adverse effects that outweigh potential benefits in GERD treatment [33]. Attempts to develop other TLESR-inhibiting drugs have been unsuccessful to date, that is why proton pump inhibitors (PPIs) remain the cornerstone of GERD treatment to date. PPIs reduce the acidity of the refluxate, and thus relieve symptoms and cure esophagitis. The efficacy of PPI therapy largely depends on bioavailability which is dependent on metabolism by cytochrome P450 (CYP) 2C19, the biological activity of which is dependent on its polymorphic genetic code [34]. Chapter 5 describes prevalence of different genotypes of this enzyme and its role in treatment received by GERD patients.

Besides inhibition of acid secretion with PPIs or histamine receptor 2 antagonists gastric contents can briefly be neutralized with antacids. Furthermore gastric emptying can be accelerated with prokinetics, sometimes providing relief, furthermore several compounds are available that form a protective layer on the esophageal mucosa. Visceral sensitivity has been shown to react to several agents (e.g. selective serotonin reuptake inhibitors) in experimental settings [35-37], however, the therapeutic application remains off-label to date.

When medical therapy is insufficient, surgical therapy can provide relief. Despite persistent controversy, evidence shows that fundoplication can provide long-term relief for GERD patients who are refractory under maximum PPI therapy or who do not want to use acid suppressants for the rest of their lives [38]. The long-term results of endoscopically applied enhancement of LES function have been disappointing [39, 40].

Genetics

Familial clustering studies [41, 42] and twin studies [43] suggest that a considerable hereditary tendency exists towards the development of GERD. This has been shown for GERD symptoms, hiatus hernia, esophagitis and Barret's alike. Genetic association studies have tied the presence of Barrett's esophagus to several functional genetic polymorphisms [44]. Testing groups of patients and controls for differences in prevalence of functional genetic polymorphisms with putative roles in the pathophysiology of the disease that is studied is called the "candidate gene"-approach, this approach is employed in chapters 6 and 7. A variation of as little as one nucleotide in the genetic code can cause a difference in the functionality of the protein it codes for. The single nucleotide polymorphism (SNP) in the

GNB3 gene [45], which is investigated in chapter 6, is a good example of how such a small difference can work to alter the functionality of a protein. In a minority of people, the cytosine (C) at position 825 is substituted for a thymine (T). Figure 1. is a schematic representation of a G-protein-coupled receptor (GPCR) and the difference between the product of the wild-type 825C gene and the product of the 825T gene.

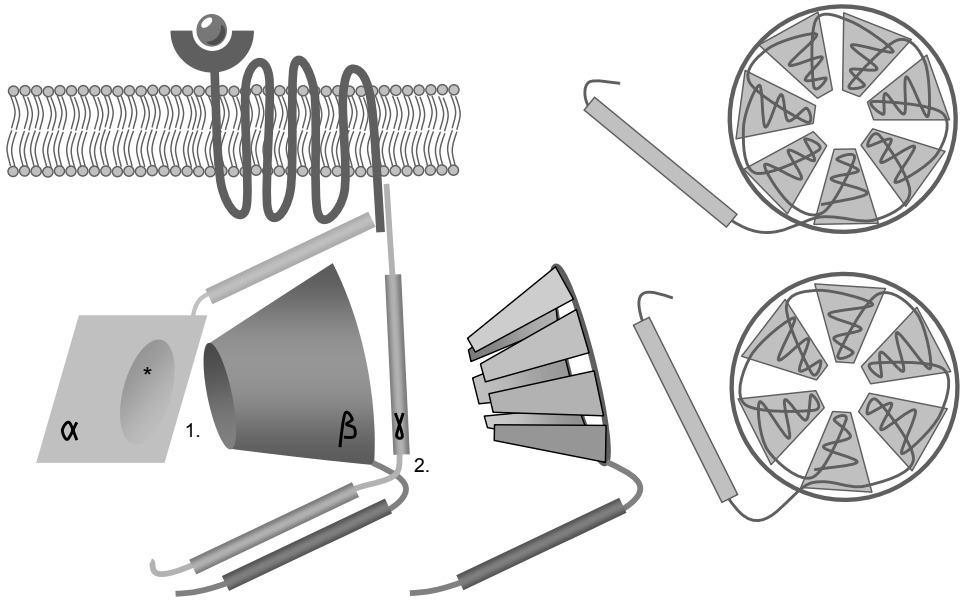


Figure 1: the GNB3 825 C/T polymorphism

In this schematic representation, a generic G-protein coupled receptor (GPCR) is depicted, recognizable by the seven transmembrane domains. Intracellular anchor sites bind the G-protein, which consists of an alpha, a beta and a gamma subunit. The most common, or "wild-type" GNB3 gene results in a protein with seven beta-pleated sheets folded into a funnel, and an alpha-helix containing tail. Upon binding of substrate to the GPCR, the beta-gamma dimer detaches from the alpha subunit, after which both remnants are active second messengers. The variant GNB3 825T results in a protein with six sheets in the funnel instead of seven, and as a result this protein is less specific in gamma-subunit binding (1.) and detaches more easily (2.). T carriers predominantly express the variant protein, resulting in enhanced signal transduction at many sites.

The wild type gene translates into a protein with one alpha-helix and seven beta-pleated sheets which are arranged into a seven-bladed fan formation. The beta-3 subunit which is formed after translation of the variant gene lacks one beta-pleated sheet, resulting in a six-bladed fan. Despite the major deletion, the protein functions. Three mechanisms are responsible for phenotypic differences between the CC genotype and the CT and TT genotypes. Firstly, in T-carriers the variant protein is more abundant than the wild-type protein. Secondly, the variant subunit is less specific in the formation of dimers with the gamma-subunit (figure 1; 1.). Moreover, the bond between the variant protein and the alpha-subunit is weaker, and as a result the beta-gamma heterodimers containing the variant protein detach more easily and transduce signals earlier (figure1; 2.) [46, 47].

In chapter 7, three genetic polymorphisms in the serotonergic signaling pathway are evaluated. These serve well to illustrate the diversity of functional genetic polymorphisms: one is an insertion/deletion promoter polymorphism [48], another a promoter SNP [49], both influencing transcription, and the third is an upstream open reading frame SNP, influencing mRNA translation [50].

A second approach to discover genetic mechanisms underlying pathophysiology is based on the comparison of the mRNA content of tissue samples. In chapters 8 and 9, genome-wide mRNA expression analysis is used to investigate differences in the transcriptome from esophageal mucosal biopsies and duodenal mucosal biopsies, respectively. Esophageal mucosa from GERD patients has been the subject of several studies, showing upregulation of several pro-inflammatory substances in inflamed and non-inflamed mucosa [51, 52]. The gene expression patterns in duodenal mucosa of GERD patients have not been investigated before.

The investigatory approach which led to chapters 8 and 9 is fundamentally different from the candidate gene approach mentioned above. Instead of comparing one carefully chosen trait between two groups, the expression of every human gene was measured and compared using microarray chips. In this setup, it was expected that most of the genes would not have different expression levels. The approach was chosen to enable the discovery of hitherto unknown mechanisms, situated in the esophageal and duodenal mucosa, playing a role in GERD pathophysiology.

Translational medicine

Translational medicine is the process through which basal scientific discoveries traverse through experimental and clinical studies towards applications in patient care. The completion of the human genome project marks a pivotal development in the field of translational medicine. The constantly growing body of evidence concerning gene functions and hereditary traits has made it possible to conceptualize and actually produce laboratory tools that bring genome-wide screening methods within the grasp of every researcher.

In the present thesis, GERD patients are compared to control subjects with regard to genetic make-up, gene expression, gastroesophageal function and visceral sensitivity, anthropometry, sociodemographic and medical background and health-related quality of life.

References

1. Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. Am J Gastroenterol 2006; 101(8):1900-1920.

2. Dent J, El Serag HB, Wallander MA, Johansson S. Epidemiology of gastrooesophageal reflux disease: A systematic review. Gut 2005; 54(5):710-717.

3. Ronkainen J, Aro P, Storskrubb T 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):275-285.

4. Zagari RM, Fuccio L, Wallander MA et al. Gastro-oesophageal reflux symptoms, oesophagitis and Barrett's oesophagus in the general population: the Loiano-Monghidoro study. Gut 2008; 57(10):1354-1359.

5. Locke GR, Zinsmeister AR, Fett SL, Melton LJ, Talley NJ. Overlap of gastrointestinal symptom complexes in a US community. Neurogastroent Motil 2005; 17(1):29-34.

6. Ronkainen J, Aro P, Storskrubb T et al. Gastro-oesophageal reflux symptoms and health-related quality of life in the adult general population - the Kalixanda study. Aliment Pharmacol Ther 2006; 23(12):1725-1733.

7. Orlando RC. oesophageal mucosal resistance. Aliment Pharmacol Ther 1998; 12(3):191-197.

8. Holloway RH. The anti-reflux barrier and mechanisms of gastro-oesophageal reflux. Best Pract Res Clin Gastroenterol 2000; 14(5):681-699.

9. Gordon C, Kang JY, Neild PJ, Maxwell JD. The role of the hiatus hernia in gastro-oesophageal reflux disease. Aliment Pharmacol Ther 2004; 20(7):719-732.

10. Ott DJ, Glauser SJ, Ledbetter MS, Chen MY, Koufman JA, Gelfand DW. Association of hiatal hernia and gastroesophageal reflux: correlation between presence and size of hiatal hernia and 24-hour pH monitoring of the esophagus. Am J Roentgenol 1995; 165(3):557-559.

11. Van Herwaarden M, Samsom M, Smout A. Excess gastroesophageal reflux in patients with hiatus hernia is caused by mechanisms other than transient LES relaxations. Gastroenterology 2000; 119(6):1439-1446.

12. Bredenoord AJ, Weusten BL, Timmer R, Smout AJ. Intermittent spatial separation of diaphragm and lower esophageal sphincter favors acidic and weakly acidic reflux. Gastroenterology 2006; 130(2):334-340.

13. Wilson LJ, Ma W, Hirschowitz BI. Association of obesity with hiatal hernia and esophagitis. Am J Gastroenterol 1999; 94(10):2840-2844.

14. Jacobson BC, Somers SC, Fuchs CS, Kelly CP, Camargo CA. Body-mass index and symptoms of gastroesophageal reflux in women. N Engl J Med 2006; 354(22):2340-2348.

15. Wu JC, Mui LM, Cheung CM, Chan Y, Sung JJ. Obesity is associated with increased transient lower esophageal sphincter relaxation. Gastroenterology 2007; 132(3):883-889.

16. El-Serag HB, Ergun GA, Pandolfino J, Fitzgerald S, Tran T, Kramer JR. Obesity increases oesophageal acid exposure. Gut 2007; 56(6):749-755.

17. El Serag HB, Tran T, Richardson P, Ergun G. Anthropometric correlates of intragastric pressure. Scand J Gastroenterol 2006; 41(8):887-891.

18. Corley DA, Kubo A, Levin TR et al. Abdominal obesity and body mass index as risk factors for Barrett's esophagus. Gastroenterology 2007; 133(1):34-41.

19. Bredenoord AJ, Weusten BLAM, Curvers WL, Timmer R, Smout AJPM. Determinants of perception of heartburn and regurgitation. Gut 2006; 55(3):313-318.

20. Emerenziani S, Sifrim D, Habib FI et al. Presence of gas in the refluxate enhances reflux perception in non-erosive patients with physiological acid exposure of the oesophagus. Gut 2008; 57(4):443-447.

21. Sarkar S, Thompson DG, Woolf CJ, Hobson AR, Millane T, Aziz Q. Patients with chest pain and occult gastroesophageal reflux demonstrate visceral pain hypersensitivity which may be partially responsive to acid suppression. Am J Gastroenterol 2004; 99(10):1998-2006.

22. 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.

23. Trimble KC, Farouk R, Pryde A, Douglas S, Heading RC. Heightened visceral sensation in functional gastrointestinal disease is not site-specific. Evidence for a generalized disorder of gut sensitivity. Dig Dis Sci 1995; 40(8):1607-1613.

24. Sarkar S, Aziz Q, Woolf CJ, Hobson AR, Thompson DG. Contribution of central sensitisation to the development of non-cardiac chest pain. Lancet 2000; 356(9236):1154-1159.

25. Meyer JH, Lembo A, Elashoff JD, Fass R, Mayer EA. Duodenal fat intensifies the perception of heartburn. Gut 2001; 49(5):624-628.

26. Boeckxstaens GE. The lower oesophageal sphincter. Neurogastroent Motil 2005; 17:13-21.

27. Stacher G, Lenglinger J, Eisler M et al. Esophageal acid exposure in upright and recumbent postures: roles of lower esophageal sphincter, esophageal contractile and transport function, hiatal hernia, age, sex, and body mass. Dig Dis Sci 2006; 51(11):1896-1903.

28. Weusten BL, Roelofs JM, Akkermans LM, Berge-Henegouwen GP, Smout AJ. The symptom-association probability: an improved method for symptom analysis of 24-hour esophageal pH data. Gastroenterology 1994; 107(6):1741-1745.

29. Wiener GJ, Richter JE, Copper JB, Wu WC, Castell DO. The Symptom Index - A Clinically Important Parameter of Ambulatory 24-Hour Esophageal Ph Monitoring. Am J Gastroenterol 1988; 83(4):358-361.

30. Galmiche JP, Clouse RE, Balint A et al. Functional esophageal disorders. Gastroenterology 2006; 130(5):1459-1465.

31. Aanen MC, Weusten BL, Numans ME, De Wit NJ, Baron A, Smout AJ. Diagnostic value of the proton pump inhibitor test for gastro-oesophageal reflux disease in primary care. Aliment Pharmacol Ther 2006; 24(9):1377-1384.

32. Smout AJ. The patient with GORD and chronically recurrent problems. Best Pract Res Clin Gastroenterol 2007; 21(3):365-378.

33. Herwaarden MA, Samsom M, Rydholm H, Smout AAJP. The effect of baclofen on acid reflux and reflux mechanisms in patients with gastroesophageal reflux disease. Gastroenterology 2001; 120(5):A433.

34. Sagar M, Tybring G, Dahl ML, Bertilsson L, Seensalu R. Effects of omeprazole on intragastric pH and plasma gastrin are dependent on the CYP2C19 polymorphism. Gastroenterology 2000; 119(3):670-676.

35. 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):365-370.

36. Holzer P. Sensory neurone responses to mucosal noxae in the upper gut: relevance to mucosal integrity and gastrointestinal pain. Neurogastroent Motil 2002; 14(5):459-475.

37. Friedrich AE, Gebhart GF. Modulation of visceral hyperalgesia by morphine and cholecystokinin from the rat rostroventral medial medulla. Pain 2003; 104(1-2):93-101.

38. Draaisma WA, Rijnhart-De Jong HG, Broeders IA, Smout AJ, Furnee EJ, Gooszen HG. Five-year subjective and objective results of laparoscopic and conventional Nissen fundoplication: a randomized trial. Ann Surg 2006; 244(1):34-41.

39. Schiefke I, Zabel-Langhennig A, Neumann S, Feisthammel J, Moessner J, Caca K. Long term failure of endoscopic gastroplication (EndoCinch). Gut 2005; 54(6):752-758.

40. Schiefke I, Neumann S, Zabel-Langhennig A, Moessner J, Caca K. Use of an endoscopic suturing device (the "ESD") to treat patients with gastroesophageal reflux disease, after unsuccessful EndoCinch endoluminal gastroplication: another failure. Endoscopy 2005; 37(8):700-705.

41. Romero Y, Cameron AJ, Locke GR, III et al. Familial aggregation of gastroesophageal reflux in patients with Barrett's esophagus and esophageal adenocarcinoma. Gastroenterology 1997; 113(5):1449-1456.

42. Trudgill NJ, Kapur KC, Riley SA. Familial clustering of reflux symptoms. Am J Gastroenterol 1999; 94(5):1172-1178.

43. Mohammed I, Cherkas LF, Riley SA, Spector TT, Trudgill NJ. Genetic influences in gastro-oesophageal reflux disease: a twin study. Gut 2003; 52(8):1085-1089.

44. Moons LM, Kusters JG, van Delft JH et al. A pro-inflammatory genotype predisposes to Barrett's esophagus. Carcinogenesis 2008; 29(5):926-931.

45. Siffert W, Rosskopf D, Siffert G et al. Association of a human G-protein beta3 subunit variant with hypertension. Nat Genet 1998; 18(1):45-48.

46. Rosskopf D, Manthey I, Habich C et al. Identification and characterization of G beta 3s2, a novel splice variant of the G-protein beta 3 subunit. Biochemical Journal 2003; 371:223-232.

47. Rosskopf D, Koch K, Habich C et al. Interaction of G beta 3s, a splice variant of the G-protein G beta 3, with G gamma- and G alpha-proteins. Cellular Signalling 2003; 15(5):479-488.

48. Lesch KP, Bengel D, Heils A et al. Association of Anxiety-Related Traits with a Polymorphism in the Serotonin Transporter Gene Regulatory Region. Science 1996; 274(5292):1527-1531.

49. Van Den BA, Sleegers K, De ZS et al. Association of brain-specific tryptophan hydroxylase, TPH2, with unipolar and bipolar disorder in a Northern Swedish, isolated population 1. Arch Gen Psychiatry 2006; 63(10):1103-1110.

50. Niesler B, Flohr T, Nothen MM et al. Association between the 5' UTR variant C178T of the serotonin receptor gene HTR3A and bipolar affective disorder 1. Pharmacogenetics 2001; 11(6):471-475.

51. Vallbohmer D, DeMeester SR, Oh DS et al. Antireflux Surgery Normalizes Cyclooxygenase-2 Expression in Squamous Epithelium of the Distal Esophagus. Am J Gastroenterol 2006; 101(7):1458-1466.

52. Yoshida N, Uchiyama K, Kuroda M et al. Interleukin-8 expression in the esophageal mucosa of patients with gastroesophageal reflux disease. Scand J Gastroenterol 2004; 39(9):816-822.

Chapter 2.

Concomitant functional dyspepsia and irritable bowel syndrome decrease health-related quality of life in gastroesophageal reflux disease

DR de Vries
MA van Herwaarden
A Baron
AJPM Smout
M Samsom

Scandinavian Journal of Gastroenterology 42(8):951-956

ABSTRACT

Background: Previous studies reported overlap between gastroesophageal reflux symptoms, functional dyspepsia (FD) and irritable bowel syndrome (IBS). We aimed to investigate the prevalence of FD and IBS in GERD and their effect on health-related quality of life (HRQoL).

Methods: In 215 referred and 48 non-referred (non-care-seeking) GERD patients, proven with 24-hour pH-metry, FD and IBS prevalence and HRQoL were assessed with questionnaires. HRQoL in 131 matched controls was used for comparison.

Results: In this group of GERD patients 25% had FD (Dutch general population 13-14%). Thirty-five percent had IBS (Dutch general population 0.6-6%). Five percent had both FD and IBS. Only 35% had neither FD nor IBS. Among referred GERD patients FD and IBS prevalence was higher (P=0.002 vs. non-referred). Compared to controls GERD patients without FD/IBS scored lower HRQoL on only one of the nine SF-36 subscales (P≤0.001). GERD+FD scored lower on six subscales (P≤0.0005). GERD+IBS patients scored lower on eight subscales (P<0.0005). GERD+FD+IBS patients scored lower on seven subscales (P≤0.001). Compared to patients with only GERD, GERD+FD patients scored lower on five subscales (P≤0.001). GERD+IBS patients scored lower on eight subscales (P<0.0005). GERD+FD+IBS patients scored lower on six subscales (P≤0.001).

Conclusions: In patients with proven GERD, FD and IBS are more prevalent than in the general population. This prevalence is higher among care-seeking GERD patients. Only those GERD patients with concomitant FD/IBS have a much lower HRQoL. This suggests that in GERD, when properly treated, HRQoL is affected mainly by concomitant functional disorders and not by GERD itself.

INTRODUCTION

Gastroesophageal reflux disease (GERD) is defined either by the primary symptoms heartburn and acid regurgitation [1] or by 24-hour esophageal intraluminal pH-metry [2]. Patients with heartburn and acid regurgitation commonly report additional symptoms, e.g. non-cardiac chest pain, hoarseness, chronic cough, belching and complaints of a dyspeptic nature[3]. Symptoms suggestive of gastroesophageal reflux are very common in western society. The prevalence of typical GERD symptoms, occurring weekly or more frequent, is reported to range from 10-20%[4]. Previous studies reported a considerable overlap between upper and lower gastrointestinal symptoms [5-10] as well as a higher prevalence of irritable bowel syndrome (IBS) among patients with GER complaints [11]. The presence of either GERD (especially when nocturnal reflux symptoms are present) or functional gastrointestinal syndromes like functional dyspepsia (FD) and irritable bowel syndrome (IBS) has a negative effect on the health-related quality of life (HRQoL) [3, 12-16].

The overlap of upper and lower gastrointestinal symptoms, or, in other words, the alleged increased prevalence of other gastrointestinal symptoms in patients with typical gastroesophageal reflux complaints, has never been investigated in patients with proven GERD, i.e. established with 24-hour pH-metry. Furthermore, the impact on HRQoL of these additional complaints in the GERD patient population is unclear. The reported overlap between GERD and lower gastrointestinal symptoms raises the question whether it is only the actual reflux disease that leads to a decreased quality of life. It is conceivable that the lower gastrointestinal symptoms provide a substantial additional burden, which may even be even more significant.

The primary aim of the present study, therefore, was to determine the prevalence of FD and IBS in a group of patients with GERD as diagnosed with 24-hour intra-esophageal pH monitoring. The secondary aim was to determine the HRQoL of GERD patients in comparison with symptom-free controls, and to assess the additional impact of the presence of FD and/or IBS.

MATERIALS AND METHODS

Patients

263 GERD patients (161 male, mean age 50.6 yrs (range 19 – 85)) with heartburn, regurgitation or non-cardiac chest pain participated in this study, most of these, 215 patients,

were referred to our tertiary referral center, and 48 patients were recruited through a newspaper advertisement. The latter had not consulted their physician for their GERD symptoms and can be regarded as non-care-seeking GERD patients. These patients were PPI-naive. The 215 patients who were referred to our hospital can be regarded as care-seeking GERD patients. GERD was proven using the gold standard, 24-hour esophageal pH recording as described in earlier publications[17]. Pathological reflux was defined as esophageal pH below 4 for ≥ 6% of the time, and/or a positive symptom association score for heartburn or regurgitation (i.e. SI ≥ 50%[18] or SAP ≥ 95%[19]). Patients were excluded if they had any history of disease and/or surgery that affects gastrointestinal motility or gastric acid secretion, at the time of the pH monitoring.

Symptom-free volunteers

131 age- and sex-matched symptom-free volunteers (70 male, mean age 44.4 yrs (range 18.2 – 64.7), recruited by newspaper advertisement, were included in the study. They had neither history of gastrointestinal disease, nor any symptoms that pointed in that direction and no medical history and/or medication use that affected gastrointestinal motility and/or gastric acid secretion.

Questionnaires

All patients and symptom-free subjects completed a 7-item questionnaire based on the Rome-II criteria for functional dyspepsia and IBS[20].

Functional Dyspepsia Diagnosis

Functional dyspepsia symptoms and the relation to defecation were assessed with three questions. FD was defined as a sensation of pain or discomfort centered in the upper abdomen for at least 12 weeks in the preceding 12 months which needed not be consecutive. These symptoms should not be relieved exclusively by defecation or associated with the onset of a change in stool frequency or stool form, in other words: the symptoms should not be suggestive of IBS.

IBS Diagnosis

The four questions about IBS diagnosis addressed abdominal pain or discomfort for at least twelve weeks in the past twelve months, which did not need to be consecutive, the alleviation of this pain or discomfort by defecation, the change in frequency of bowel movements and the changes in consistency of stool in association with onset and/or lessening of pain or discomfort. Two of these last three aspects had to be present to diagnose IBS.

Health-Related Quality of Life

In addition, all patients and symptom-free subjects completed the RAND-36 questionnaire. This is a validated Dutch translation of the 36-item internationally validated general health-related quality of life (HRQoL) questionnaire SF-36 [21-23]. This instrument measures general quality of life divided into nine domains. These domains are: physical functioning, social functioning, physical and emotional role limits (i.e. the extent of limitation of daily activities caused by either physical or emotional problems, respectively), mental health, vitality, bodily pain, general health experience and health change.

Each of these subscales has a score ranging from 0 –100, with a score of 100 representing no impairment or maximal HRQoL. This questionnaire was used to determine the impact on HRQoL of GERD and the functional gastrointestinal disorders that were assessed.

The self-reported HRQoL in the group of 131 symptom-free volunteers was used to compare the HRQoL scores in the patient group and subgroups.

Statistical analysis

Age and gender distribution between groups were tested with one-way ANOVA and chi square tests, respectively. The prevalence of functional gastrointestinal disorders among care-seekers and non-care-seekers, males and females and patients with pathological and physiological reflux were compared using the chi square test.

Rand-36 subscale scores were calculated for five groups: symptom-free volunteers, GERD patients who had only GERD (i.e. without concomitant functional abdominal disorders), GERD patients who also suffered from FD, GERD patients who also fulfilled the criteria for IBS, and GERD patients who had both FD and IBS. Results of the RAND-36 were tested for normality with the Kolmogorov-Smirnov test. Separate statistical analysis was performed for all nine subscales of the measure. Differences between subscale scores of the five symptom groups listed above were tested with Kruskall-Wallis tests. Subsequently, the analysis was repeated for the scores of the four patient groups, leaving out the scores of symptom-free volunteers. Mean ranks were then compared using Mann-Whitney U-tests in two directions: first, the scores per scale in the four subgroups were compared to the symptom-free volunteers' subscale scores. Furthermore, the subscale scores in the patient groups with FD, IBS and both FD and IBS were compared to the scores in the group that had only GERD. Because of the large number of comparisons made concerning the HRQoL, after Bonferroni correction $P \leq 0.001$ was chosen as the border of significance for this analysis.

RESULTS

Prevalence of functional gastrointestinal disorders

The prevalence of FD and IBS in the total GERD patient group is depicted in table 1. Overall, 65% of the patients had a concomitant functional disorder. There were no differences in prevalence of these disorders when male patients were compared to female patients.

Table 1: Quality of life as scored on RAND-36 by healthy volunteers and patients

	Healthy	GERD only	GERD & FD	GERD & IBS	GERD, FD & IBS
Group Size	131	91 (35%)	66 (25%)	93 (35%)	13 (5%)
Age	44 (18 – 65)	50 (20 – 85)	52 (23 – 74)	51 (19 – 79)	49 (38 – 68)
N° of males	70 (53%)	65 (71%)	42 (64%)	51 (55%)	3 (23%)
SF-36 subscale					
Physical functioning	100 (10.0) 60 – 100	95.0 (20.0)* 10 – 100	85.0 (35.0)**† 15 – 100	65.0 (42.5) **‡ 5 – 100	60.0 (25.0) **‡ 0 – 100
Social functioning	100 (12.5) 37.5 – 100	100 (25.0) 25 – 100	81.3 (37.5) **† 12.5 – 100	62.5 (37.5) **‡ 0 – 100	62.5 (56.3)** 37.5 – 100
Role Physical	100 (0.0) 0 – 100	100 (0.0) 0 – 100	87.5 (75.0)** 0 – 100	25.0 (100) **‡ 0 – 100	25.0 (100) **† 0 – 100
Role Emotional	100 (0.0) 0 – 100	100 (0.0) 0 – 100	100 (8.3) 0 – 100	100 (83.4) **‡ 0 – 100	66.7 (100) **‡ 0 – 100
Mental Health	80.0 (16.0) 20 – 100	80.0 (16.0) 32 – 100	76.0 (20.0) 24 – 96	68.0 (28.0) **‡ 16 – 100	68.0 (24.0) **‡ 24 – 100
Vitality	75.0 (20.0) 30 – 100	70.0 (25.0) 30 – 100	60.0 (26.3) **‡ 10 – 90	45.0 (32.5) **‡ 10 – 90	45.0 (25.0) 30 – 85
Bodily Pain	100 (10.2) 20.4 – 100	89.8 (30.6) 22.4 – 100	67.3 (32.7) **‡ 10.2 – 100	57.1 (28.6) **‡ 10.2 – 100	44.9 (23.5) **‡ 10.2 – 100
General Health	80.0 (20.0) 50 – 100	75.0 (30.0) 10 – 100	60.0 (40.0) **‡ 0 – 95	45.0 (35.0) **‡ 0 – 100	50.0 (30.0) **‡ 15 – 75
Health Change	50.0 (0.0) 25 – 100	50.0 (25.0) 0 – 100	50.0 (50.0) 0 – 100	50.0 (50.0) 0 – 100	50.0 (50.0) 0 – 100

Group sizes are displayed as N (%); ages are displayed as mean (range); Sex is displayed as number of males (%); RAND-36 Subscale scores are displayed as median (interquartile range) with the range in the row below. * P=0.001 compared to healthy volunteers; ** P<0.001 compared to healthy volunteers † P=0.001 compared to "GERD only"; ‡ P<0.001 compared to "GERD only"

Distribution of functional abdominal disorders amongst referred and non-referred GERD patients

Functional abdominal disorders were more prevalent in the care-seeking group than in the non-care-seeking group, as displayed in table 2.

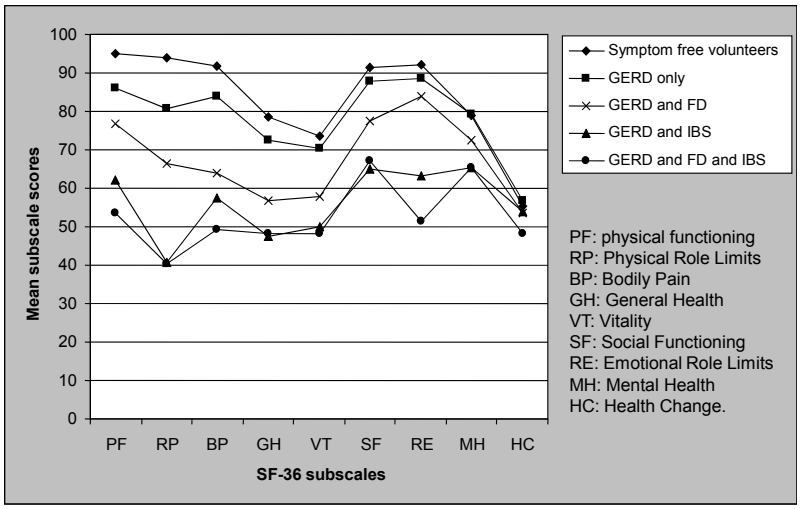
Table 2. Prevalence of FD and IBS in the care-seeking and non-care-seeking group.

	N	Male	Age	GERD	GERD & FD	GERD & IBS	GERD, FD & IBS
Care-seeking	215	128 (60)	50 (19 – 85)	65 (30)	56 (26)	83 (39)	11 (5)
Non-care-seeking	48	33 (69)	55 (28 – 78)	26 (54)	10 (21)	10 (21)	2 (4)

Sex is displayed as number of males (%); age is displayed as mean (range); subgroups are displayed as N (%). In the non-care-seeking group, more patients had only GERD without concomitant FD and/or IBS (P=.002).

In the care-seeking group 30% of the patients had no concomitant functional gastrointestinal disorder, in the non-care-seeking group 54% of patients suffered from GERD only (P=0.002). Gender distribution was comparable in both groups.

Figure 1: Mean subscale scores in the five investigated groups.



HRQoL

Median subscale scores and accompanying P-values are summarized in table 1 and a visual representation of these data is given in the figure, which depicts mean subscale scores per subject group. In general, the scores were highest in the symptom-free volunteer group, followed by the scores of the patient group with GERD only. The groups with concomitant functional disorders scored lower on most subscales. An exception was the "health change" scale, the score which reflects the perceived change in general health in the past year: all five group means were close to 50, meaning: no change. The results for the remaining eight subscales will be summarized below.

Comparison of scores on the eight subscales for the five subgroups all yielded P-values below 0.001. For the four patient groups the scores for all eight subscales were significantly different ($P<0.001$) as well.

GERD patients who did not meet the criteria for FD and/or IBS did not score significantly lower than symptom-free controls on most subscales. Only on the *physical functioning* scale did the difference reach statistical significance. In contrast, in the group that had FD besides their GERD, almost all subscale scores were not only lower than those of symptom-free controls, but also than those of patients that had only GERD. When compared to controls, no significant impairment in *emotional role limits* and *mental health* was found. When compared to patients who had only GERD, this group showed the same impairments with the marginal exception of the *physical role limits* scale. (Thus, the patients with GERD and FD experienced impairment of their quality of life on more levels than patients with GERD only.)

In the group that had IBS besides GERD, impairment was significant on all scales when compared to controls as well as to patients with only GERD.

The small group of patients that, in addition to GERD, had both IBS and FD reported significant impairment on the same scales when compared to symptom-free volunteers with the exception of *vitality*. Compared to patients who had only GERD this group scored lower on six out of eight subscales.

DISCUSSION

This study shows that in GERD patients whose reflux disease has been objectively proven using 24-hour pH-metry there is a high prevalence of both functional dyspepsia and irritable bowel syndrome: 25% and 35%, respectively. In comparison, the reported FD prevalence in

the Netherlands is 13 to 14% [24, 25], the reported prevalence for IBS in the Dutch society ranges from 0.6 to 6% [26]. Only 35% of our GERD patients do not qualify for either FD or IBS. In the subset of patients who seek medical care the prevalence of these syndromes is higher than in the group of GERD patients who were recruited by advertisements and did not seek medical care. However, the prevalence in the non-care-seeking group is still high, nearly half the patients have a concomitant functional disorder. These findings suggest that the presence of FD or IBS does not play a pivotal role in care-seeking behavior. Note that the present study did not yield details about the severity and frequency of the symptoms that constitute FD and IBS. Ahlawat et al. showed that these parameters are the strongest predictors of health-care-seeking in patients with any type of dyspepsia [27].

Our findings indicate that FD and IBS negatively affect the health-related quality of life of GERD patients. GERD patients without FD or IBS reported lower quality of life than symptom-free subjects, but only on the *physical functioning* subscale of the RAND-36 questionnaire. We found that patients who had a concomitant functional gastrointestinal syndrome reported lower quality of life on most of the subscales in the presence of FD and all subscales in the presence of IBS. The GERD patients that had both FD and IBS scored as low as the GERD patients with IBS, or lower.

The scores for one specific subscale (*health change*) are strikingly similar: none of the groups reported a significant change in general health in the past year. The chronic nature of both GERD and functional gastrointestinal disorders can very well explain this finding [28].

When the current data are compared to data from the general Dutch population as published by Aaronson et al. [29] it is clear that the symptom-free group's scores are consistent with the absence of gastrointestinal complaints and severe comorbidity that was required for inclusion. Age and disease in all fields lower the scores in the general population. In the group with only GERD, scores are seen that approach those of the general population in most domains. When concomitant FD and/or IBS are present, HRQoL scores sink under the level of the general population.

These findings confirm the outcomes of previous studies, in which history-derived criteria were used to establish a diagnosis of GERD. Pimentel et al. found a higher prevalence of IBS among GERD patients, however the study was limited by a poor response rate and a relatively small group of patients [11]. Locke et al. reported an overlap between upper and lower gastrointestinal symptom complexes [30]. Talley et al. found that in IBS patients an overlap existed between upper and lower gastrointestinal symptoms [10].

The results of this study and the studies referred to above suggest that a generalized disorder of visceral sensitivity might be involved in the pathogenesis of GERD, FD and IBS.

Evidence for this has not only been established epidemiologically as in the studies mentioned above. Sarkar et al. found that baseline esophageal pain thresholds were lower in GERD patients than in symptom-free volunteers. Moreover, pain thresholds in the upper esophagus of symptom-free volunteers was lowered by infusion of acid in the lower esophagus, but in GERD patients this effect was not seen, pointing towards visceral pain hypersensitivity [31]. In several studies altered visceral perception was found on more than one location in the intestinal tract. Trimble et al. found lower pain thresholds in the esophagus and stomach of IBS patients, and also in the esophagus and rectum of FD patients compared to symptom-free controls [32]. Corsetti et al. found that FD patients who had coexisting IBS were more sensitive to distention of the stomach than FD patients who did not fulfill the criteria for IBS [33].

The findings made in the present study provide a further argument in favor of a common denominator in functional gastrointestinal disorders. Even GERD, a disorder that can be quantified with pH monitoring, cannot be seen as an isolated disease. When no other symptom complexes are present and the reflux symptoms are treated according to standards, the self-reported health-related quality of life is not significantly lower than the HRQoL of an age- and sex-matched group of symptom-free volunteers. Only when other disorders, like FD and IBS in the current report, are present, HRQoL starts to decrease considerably. This corroborates the idea that visceral hypersensitivity is more detrimental than the reflux itself, provided that the latter is treated adequately.

One could perhaps expect that the prevalence of FD and IBS in the subgroup of GERD patients who have a physiological reflux time with a positive symptom association is higher than in the group of patients with pathological reflux. This was not observed in the current study. Proof of the relation between visceral hypersensitivity and GERD has been provided sufficiently. However, the pathophysiological mechanism and the direction of cause and effect remain to be elucidated.

In summary, FD and IBS are more prevalent among patients with proven GERD, and the quality of life of GERD patients is strikingly lowered by the concomitant presence of either FD or IBS or by the presence of both. The results of the present study suggest that HRQoL in properly treated GERD patients is decreased more by concomitant functional abdominal complaints than by GERD itself.

References

1. Holloway RH. The anti-reflux barrier and mechanisms of gastro-oesophageal reflux. Best Pract Res Clin Gastroenterol 2000; 14(5):681-699.

2. Locke GR, Talley NJ. 24-hour monitoring for gastro-oesophageal reflux disease. Lancet 1993; 342(8881):1246-1247.

3. Talley NJ. Review article: gastro-oesophageal reflux disease -- how wide is its span? Aliment Pharmacol Ther 2004; 20 Suppl 5:27-37.

4. Dent J, El Serag HB, Wallander MA, Johansson S. Epidemiology of gastrooesophageal reflux disease: A systematic review. Gut 2005; 54(5):710-717.

5. Locke GR, Zinsmeister AR, Fett SL, Melton LJ, Talley NJ. Overlap of gastrointestional symptom complexes in a US community. Neurogastroent Motil 2005; 17(1):29-34.

6. Tack J, Caenepeel P, Arts J, Lee KJ, Sifrim D, Janssens J. Prevalence of acid reflux in functional dyspepsia and its association with symptom profile. Gut 2005; 54(10):1370-1376.

7. Talley NJ, Piper DW. The association between non-ulcer dyspepsia and other gastrointestinal disorders. Scand J Gastroenterol 1985; 20(7):896-900.

8. Talley NJ, Boyce P, Jones M. Identification of distinct upper and lower gastrointestinal symptom groupings in an urban population. Gut 1998; 42(5):690-695.

9. Talley NJ, Holtmann G, Agreus L, Jones M. Gastrointestinal symptoms and subjects cluster into distinct upper and lower groupings in the community: a four nations study. Am J Gastroenterol 2000; 95(6):1439-1447.

10. Talley NJ, Dennis EH, Schettler-Duncan VA, Lacy BE, Olden KW, Crowell MD. Overlapping upper and lower gastrointestinal symptoms in irritable bowel syndrome patients with constipation or diarrhea. Am J Gastroenterol 2003; 98(11):2454-2459.

11. Pimentel M, Rossi F, Chow EJ et al. Increased prevalence of irritable bowel syndrome in patients with gastroesophageal reflux. J Clin Gastroenterol 2002; 34(3):221-224.

12. Creed F, Ratcliffe J, Fernandez L et al. Health-related quality of life and health care costs in severe, refractory irritable bowel syndrome. Ann Intern Med 2001; 134(9):860-868.

13. Eloubeidi MA, Provenzale D. Health-related quality of life and severity of symptoms in patients with Barrett's esophagus and gastroesophageal reflux disease patients without Barrett's esophagus. Am J Gastroenterol 2000; 95(8):1881-1887.

14. Revicki, Crawley, Zodet, Levine, Joelsson. Complete resolution of heartburn symptoms and health-related quality of life in patients with gastro-oesophageal reflux disease. Aliment Pharmacol Ther 1999; 13(12):1621-1630.

15. Chang L. Review article: epidemiology and quality of life in functional gastrointestinal disorders. Aliment Pharmacol Ther 2004; 20:31-39.

16. Farup C, Kleinman L, Sloan S et al. The impact of nocturnal symptoms associated with gastroesophageal reflux disease on health-related quality of life. Arch Intern Med 2001; 161(1):45-52.

17. Van Herwaarden M, Samsom M, Smout A. Excess gastroesophageal reflux in patients with hiatus hernia is caused by mechanisms other than transient LES relaxations. Gastroenterology 2000; 119(6):1439-1446.

18. Wiener GJ, Richter JE, Copper JB, Wu WC, Castell DO. The Symptom Index - A Clinically Important Parameter of Ambulatory 24-Hour Esophageal Ph Monitoring. Am J Gastroenterol 1988; 83(4):358-361.

19. Weusten BL, Roelofs JM, Akkermans LM, Berge-Henegouwen GP, Smout AJ. The symptom-association probability: an improved method for symptom analysis of 24-hour esophageal pH data. Gastroenterology 1994; 107(6):1741-1745.

20. Drossman DA (sen.ed.). ROME II: The Functional Gastrointestinal Disorders, *Second Edition*. 2 ed. 1998.

21. Hunt SM, Mckenna SP. Validating the Sf-36. Brit Med J 1992; 305(6854):645.

22. Mant JWF, Jenkinson C, Murphy MFG, Clipsham K, Marshall P, Vessey MP. Use of the Short Form-36 to detect the influence of upper gastrointestinal disease on self-reported health status. Qual Life Res 1998; 7(3):221-226.

23. Ware JE, Sherbourne CD. The Mos 36-Item Short-Form Health Survey (Sf-36) .1. Conceptual-Framework and Item Selection. Med Care 1992; 30(6):473-483.

24. Boekema PJ, van Dam van Isselt E, Bots ML, Smout AJPM. Functional bowel symptoms in a general Dutch population and associations with common stimulants. Neth J Med 2001; 59(1):23-30.

25. Schlemper RJ, VanderWerf SDJ, Vandenbroucke JP, Biemond I, Lamers CBHW. Peptic-Ulcer, Nonulcer Dyspepsia and Irritable-Bowel-Syndrome in the Netherlands and Japan. Scand J Gastroenterol 1993; 28:33-41.

26. Schryver AMP de, Samsom M. Het prikkelbaredarmsyndroom: pathofysiologie en (toekomstige) behandelingsmogelijkheden. Ned Tijdschr Geneesk 2002; 146(1):12-16.

27. Ahlawat SK, Locke GR, III, Weaver AL, Farmer SA, Yawn BP, Talley NJ. Dyspepsia consulters and patterns of management: a population-based study. Aliment Pharmacol Ther 2005; 22(3):251-259.

28. Agreus L, Svardsudd K, Talley NJ, Jones MP, Tibblin G. Natural history of gastroesophageal reflux disease and functional abdominal disorders: a population-based study. Am J Gastroenterol 2001; 96(10):2905-2914.

29. Aaronson NK, Muller M, Cohen PDA et al. Translation, validation, and norming of the Dutch language version of the SF-36 Health Survey in community and chronic disease populations. Journal of Clinical Epidemiology 1998; 51(11):1055-1068.

30. Locke GR, Zinsmeister AR, Fett SL, Melton LJ, Talley NJ. Overlap of gastrointestional symptom complexes in a US community. Neurogastroent Motil 2005; 17(1):29-34.

31. Sarkar S, Thompson DG, Woolf CJ, Hobson AR, Millane T, Aziz Q. Patients with chest pain and occult gastroesophageal reflux demonstrate visceral pain hypersensitivity which may be partially responsive to acid suppression. Am J Gastroenterol 2004; 99(10):1998-2006.

32. Trimble KC, Farouk R, Pryde A, Douglas S, Heading RC. Heightened visceral sensation in functional gastrointestinal disease is not site-specific. Evidence for a generalized disorder of gut sensitivity. Dig Dis Sci 1995; 40(8):1607-1613.

33. Corsetti M, Caenepeel P, Fischler B, Janssens J, Tack J. Impact of coexisting irritable bowel syndrome on symptoms and pathophysiological mechanisms in functional dyspepsia. Am J Gastroenterol 2004; 99(6):1152-1159.

Chapter 3.

Gastroesophageal pressure gradients in gastro-esophageal reflux disease: relations with hiatal hernia, body mass index and esophageal acid exposure

DR de Vries
MA van Herwaarden
AJPM Smout
M Samsom

American Journal of Gastroenterology 2008;103:1349-54

ABSTRACT

Background: The roles of intragastric pressure (IGP), intraesophageal pressure (IEP), gastroesophageal pressure gradient (GEPG) and BMI in the pathophysiology of gastroesophageal reflux disease (GERD) and hiatal hernia (HH) are only partly understood.
Methods: 149 GERD patients underwent stationary esophageal manometry, 24-hour pH-metry and endoscopy.
Results: 103 patients had HH. Linear regression analysis showed that each kgm^{-2} of BMI caused a 0.047 kPa increase of inspiratory IGP (95%CI 0.026-0.067) and a 0.031 kPa increase of inspiratory GEPG (95%CI 0.007-0.055). Each kgm^{-2} of BMI caused expiratory IGP to increase with 0.043 kPa (95%CI 0.025-0.060) and expiratory IEP with 0.052 kPa (95%CI 0.027-0.077). Each added year of age caused inspiratory IEP to decrease with 0.008 kPa (95%CI -0.015 – -0.001) and inspiratory GEPG to increase with 0.008 kPa (95%CI 0.000-0.015). In binary logistic regression analysis HH was predicted by inspiratory and expiratory IGP (odds ratio (OR) 2.93 and 2.62 respectively), inspiratory and expiratory GEPG (OR 3.19 and 2.68, respectively) and BMI (OR 1.72 per 5 kgm^{-2}). In linear regression analysis HH caused an average 5.09% increase in supine acid exposure (95%CI 0.96-9.22) and an average 3.46% increase in total acid exposure (95%CI 0.82-6.09). Each added year of age caused an average 0.10% increase of upright acid exposure and a 0.09% increase of total acid exposure (95%CI 0.00-0.20 and 0.00-0.18).
Conclusions: BMI predicts IGP, inspiratory GEPG and expiratory IEP. Age predicts inspiratory IEP and GEPG. Presence of HH is predicted by IGP, GEPG and BMI. GEPG is not associated with acid exposure.

INTRODUCTION

Gastroesophageal reflux disease (GERD) is very common in the western society with 10-20% of the population suffering from one or more of the cardinal symptoms at least weekly [1]. The main mechanism by which gastroesophageal reflux takes place is the transient relaxation of the lower esophageal sphincter (TLESR) [2]. In patients with hiatal hernia (HH), however, the reflux occurs through other mechanisms [3]. In order to facilitate flow of gastric contents into the esophagus, the gastroesophageal pressure gradient (GEPG) must be positive. The GEPG has been shown, both in GERD patients and healthy subjects, to be higher before and during TLESRs associated with acid reflux than during those without reflux [4, 5]. This finding was not confirmed, however, in a study in healthy subjects using intraluminal esophageal impedance monitoring, a method which detects reflux irrespective of its pH [6]. Obesity is a risk factor for the development of GERD symptoms and their severity [7-9]. A high BMI increases the GEPG mainly by raising intragastric pressure (IGP) [10, 11] but also causes a spatial separation between the pressure zone of the crural diaphragm and the LES [11]. In concordance with this observation, Wilson et al. and Stene-Larsen et al. described an association of obesity with presence of hiatal hernia [12, 13]. Moreover, in a recent report by Wu et al., a higher BMI was associated with an increased incidence of TLESRs [14].

This study aimed to integrate data on IGP, intraesophageal pressure (IEP) and GEPG, acid exposure during 24-hour intraesophageal pH-monitoring, BMI, and presence of hiatal hernia and esophagitis in a large cohort of symptomatic patients with proven GERD, in order to contribute to the understanding of the importance of these parameters in GERD pathophysiology.

METHODS

Subjects

From the records of our gastrointestinal motility research unit 149 consecutive patients were selected who had typical GERD symptoms and who were referred for 24-hour pH-metry, and had had esophageal manometry directly prior to that. Reports of upper GI endoscopy were retrieved and reviewed for the presence or absence of hiatal hernia and esophagitis, and weight and height were noted for BMI calculation. Hiatal hernia was defined as a distance of 3 cm or more between squamocolumnar junction and the impression of the crural diaphragm, measured during introduction of the endoscope. Patients with severe

concomitant disease, a history of major abdominal surgery or diseases that influence gastrointestinal motility were excluded. The protocol was approved by the medical ethics committee of our hospital.

Stationary manometry

Manometry device and placement

All patients underwent a stationary manometry directly prior to the positioning of the pH catheter. Stationary esophageal manometry was performed using a water-perfused manometric assembly with incorporated sleeve (Dentsleeve International Ltd, Mississauga, Ontario, Canada). The catheter incorporated two pharyngeal sideholes (20 and 25 cm proximal to the proximal border of the sleeve) and three esophageal sideholes (5, 10 and 15 cm proximal to the proximal border of the sleeve), a reversed perfused sleeve with one sidehole on its proximal border and one intragastric sidehole (2 cm distal to the distal border of the sleeve). Pressures were recorded with external pressure transducers (Abbott, Chicago, IL, USA). The assembly was perfused at a rate of 0.45 ml/min with degassed water using hydraulic flow restrictors (Dentsleeve International Ltd) and a water pump. The assembly was introduced through the nose until the sleeve was in the stomach. Then, the sleeve was positioned in the LES and 10 swallows of 10 ml water each were given to assess esophageal motility.

Verification of manometry catheter position

The esophageal manometry study was performed in adherence to a well-established protocol that has not changed during the period in which patients were included, and which had been in use for years before the first patient was enrolled in the study. In this routine, firstly the catheter readings were levelled in a vertical water column to minimize hydrostatic artifact. The patient was placed in a 45° reclining position. After the introduction of the catheter all sideholes and the sleeve sensor were positioned in the stomach. This gave us the opportunity to compare the recordings made by the most distal sidehole, that later would be the sidehole recording intragastric pressure, to the pressures recorded by the sidehole that would later lie in the distal esophagus. Being in the same compartment, these sideholes should record the same pressure. If they did not, the mean difference was noted and used later on for correction of the GEPG subtraction for this artifact. These correction factors, when added later on, turned out to be exactly zero and thus had no effect on the mean pressure parameters in the groups.

In order to verify that intragastric pressure was measured intra-abdominally and not in the hiatal sac of patients with HH, the mean intragastric pressure, recorded with all sideholes intragastrically positioned, was compared to the intragastric pressure that was recorded

once the other sidehole had been placed in the distal esophagus. No substantial differences between these recordings, suggestive of a position in the hiatal sac of the most distal sidehole in patients with HH, were seen. Moreover, the gastroesophageal pressure inversion point [15] was between the intragastric sidehole and the distal intraesophageal sidehole in all recordings.

Pressure assessment after positioning

Intragastric pressure was measured using the intragastric sidehole, 2 cm distal to the sleeve. The intraesophageal pressure was measured in the signal derived from the distal esophageal sidehole 5 cm proximal to the border of the sleeve. Intragastric and intraesophageal pressure were measured at 10 end-inspiratory and expiratory time points, and then averaged. GEPG was calculated, both at inspiration and at expiration, as the difference between mean intragastric and intraesophageal pressures. During ten wet swallows consisting of 10ml water esophageal contractions were assessed, and the amplitude of the distal esophageal contractions was scored as a measure of esophageal motility performance.

24-hour pH-metry

Twenty-four hour esophageal pH recordings were performed using a glass electrode with incorporated reference (model LOT 440, Ingold A.G., Urdorf, Switzerland), which was introduced transnasally and positioned 5 cm above the proximal border of the LES. Acid exposure time was defined as percentage of time with intraesophageal pH below 4. Patients were instructed to fill out diary cards regarding the time of meals and beverages, as well as recumbent time in order to be able to calculate upright and supine acid exposure time. In the analysis of the esophageal acid exposure the meals and beverages were excluded.

Statistical analysis

To investigate the relations between the various parameters, we used multiple regression analysis. To assess the relation between BMI, age and sex and intragastric and intraesophageal pressure and the GEPG a linear regression model was used with these three pressure parameters, during inspiration and expiration, as dependent variables. To assess the risk of HH, we performed binomial logistic regression analysis with presence of HH as response variable and age, sex, BMI and the six EGJ pressure parameters as predicting variables in seven separate models for reasons of strong colinearity. A multiple linear regression model was used to investigate the influence of HH, age, sex, BMI, esophageal motility performance as reflected by the distal contraction amplitude and the six

EGJ pressure parameters on the acid exposure time during 24-hour pH-metry. Binomial logistic regression analysis was used to analyze the risk of esophagitis, providing odds ratios for presence of HH, age, sex and BMI.

RESULTS

Patient characteristics

Table 1 summarizes the patient characteristics, and table 2 shows the inspiratory and expiratory IGP, IEP and GEPG in patients with and without HH. The differences between these two groups were investigated with regression analysis.

Table 1. Characteristics of patients with and without hiatal hernia

	N (M)	Age (year)	BMI	BMI category (N(%))					Esophagitis
		Mean (SD)	Mean(SD)	<20	≥20	≥25	≥30	≥35	N (%)
no HH	46 (29)	47.5 (14.4)	26.0 (3.8)	2 (4)	24 (52)	13 (28)	7 (15)	0 (0)	14 (30)
HH	103 (57)	49.3 (13.1)	27.1 (4.1)	2 (2)	29 (28)	56 (54)	13(13)	3 (3)	65 (63)

Characteristics of GERD patients with and without hiatal hernia. BMI = body mass index (kg/m²), M = male, SD = standard deviation, HH = hiatal hernia.

Table 2. EGJ pressure parameters in GERD patients with and without hiatal hernia.

	Inspiration			**Expiration**		
	IGP	IEP	GEPG	IGP	IEP	GEPG
No HH	1.53 (.063)	0.34 (.098)	1.19 (.101)	1.16 (.062)	1.02 (.099)	0.14 (.085)
HH	1.79 (.059)	0.20 (.054)	1.58 (.054)	1.35 (.045)	0.95 (.062)	0.40 (.046)

Esophagogastric junction (EGJ) pressure parameters: intragastric pressure (IGP), intraesophageal pressure (IEP) and gastroesophageal pressure gradient (GEPG) during inspiration and expiration in GERD patients with and without hiatal hernia (HH). Pressures were measured in kPa and displayed as mean (SEM).

Determinants of IGP, IEP and GEPG

Table 3 shows the results of linear regression analysis with inspiratory and expiratory IGP, IEP and GEPG as response variables. The significant slopes (=regression coefficient B) that were found indicate that a one-point increase in BMI causes inspiratory IGP to increase with 0.047 kPa and the inspiratory GEPG with 0.031 kPa, and the expiratory IGP with 0.043 kPa and the expiratory IEP with 0.052 kPa. The significant slopes found for age in years as an independent predictor indicate that each year lived decreases inspiratory IEP with 0.008 kPa and adds 0.008 kPa to the inspiratory GEPG. These models incorporated sex as covariate; sex itself however did not predict any of the pressure parameters.

Table 3. Association of body mass index and age with gastric and esophageal pressure and gastroesophageal pressure gradient

		Inspiration			Expiration		
		IGP	IEP	GEPG	IGP	IEP	GEPG
BMI	**Slope (kPa/kgm^{-2})**	**.047**	**---**	**.031**	**.043**	**.052**	**---**
	95% CI	.026 / .067		.007 / .055	.025 / .060	.027 / .077	
Age	**Slope (kPa/year)**	**---**	**-.008**	**.008**	**---**	**---**	**---**
	95% CI		-.015 / -.001	.000 / .015			
	Model R square	.128	.047	.081	.144	.107	---

Results from linear regression analysis with pressure parameters as response variables and BMI and age as predictors. IGP : intragastric pressure; IEP: intraesophageal pressure; GEPG : gastroesophageal pressure gradient ; 95% CI : confidence interval of slopes. Strong colinearity between the pressure parameters made six separate analyses necessary, hence the different R squares. Sex was added to the models as adjusting variable but showed no significant associations.

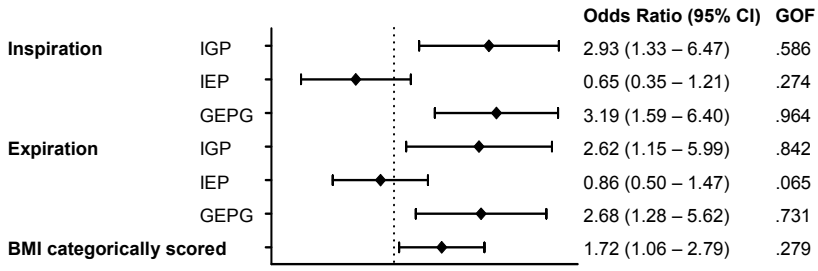
Determinants of hiatal hernia

Postulating that increased BMI, IGP and GEPG could facilitate HH, binary logistic regression analysis was performed to investigate the role of BMI, IGP, IEP and GEPG as risk factors for HH. Seven separate analyses, adjusting for age and sex, were constructed because of strong colinearity between these seven parameters. The adjusted odds ratios with 95% confidence intervals that were calculated with these models are displayed in figure 1.

Inspiratory and expiratory IGP and GEPG, as well as BMI, scored categorically (<20, 20-24.9, 25-29.9, 30-34.9, >35), were strong independent predictors of presence of HH. BMI showed no significant association with HH when it was analyzed as a continuous variable, although a trend towards significance was observed. Neither IEP, nor age, nor sex showed

any significant associations. Hosmer and Lemeshow goodness of fit P values were >0.05 for every model.

Figure 1: Association of EGJ pressure parameters and BMI with presence of hiatal hernia



Forest plot of odds ratios and 95% confidence intervals derived from binary logistic regression analysis with presence of hiatal hernia as response variable. Inspiratory and expiratory intragastric pressure (IGP), intraesophageal pressure (IEP) and gastroesophageal pressure gradient (GEPG) Age and sex were entered into the model as covariates but showed no significant associations. Strong colinearity between BMI, IGP, IEP and GEPG necessitated seven separate analyses. Hosmer Lemeshow goodness of fit (GOF) tests were performed for all seven models, P values are in the right hand column. Of note, all P values are > 0.05, ensuring goodness of fit.

Determinants of acid exposure

Table 4 shows results of linear regression analysis investigating the relationship between HH, age and esophageal motility performance as reflected by distal esophageal contraction amplitude, with sex as covariate.

The influence of IGP, IEP and GEPG on acid exposure was analyzed as well but no independent significant predicting values were observed in those models. The slopes of the significant associations with acid exposure during upright body position indicate that each added year causes in increase in upright acid exposure of 0.1%, and that each kPa of amplitude added to distal esophageal contractions decreases acid exposure in upright position with 0.28%. HH exerted no influence over acid exposure in upright position. Acid exposure during supine body position was predicted by presence of HH and contraction amplitude, the slopes indicating that HH causes an average 5.09% increase in supine acid exposure, and that each kPa of contraction amplitude causes a decrease of supine acid exposure of 0.41%. In concordance with these findings, total acid exposure during 24-hour pH-metry was predicted by HH, age and distal esophageal contraction amplitude. IGP, IEP, GEPG, BMI and sex were no predictors of acid exposure.

Table 4. Association of hiatal hernia, age and esophageal motility performance with esophageal acid exposure during 24-hour pH-monitoring

Acid exposure while:	**Upright**	**Supine**	**Total**	Unit of slope
Hiatal hernia	2.11 (-0.72 / 4.94)	**5.09 (0.96 / 9.22)**	**3.46 (0.82 / 6.09)**	Δ% in HH patient
Age	**0.10 (0.00 / 0.20)**	0.60 (-0.08 / 0.20)	**0.09 (0.00 / 0.18)**	Δ % / 1 year
Amplitude of distal esophageal contractions	**-0.28 (-0.53 / -0.04)**	**-0.41 (-0.77 / -0.06)**	**-0.34 (-0.57 / -0.12)**	Δ % / 1 kPa
Model R^2	0.09	0.08	0.13	

Slopes (B) and 95% confidence intervals resulting from linear regression analysis with esophageal acid exposure during 24-hour intraesophageal pH-monitoring as response variable. Significant associations are shown in bold type. BMI was included in the model as covariate but showed no significant associations. R^2 was used as a measure of goodness of fit. HH: hiatal hernia.

Risk factors for esophagitis

Binary logistic regression analysis was performed to investigate the risk factors for esophagitis. Eight models were constructed with HH, BMI, inspiratory and expiratory IGP, IEP and GEPG as regressors, respectively. Each model also incorporated age, sex and distal esophageal contraction amplitude as covariates. The only significant risk factor for esophagitis was HH, with an adjusted odds ratio of 4.1 (95% CI 1.9-8.7, Hosmer and Lemeshow P value 0.472).

DISCUSSION

The present study is the first to determine the relationship between intragastric and intraesophageal pressure and the gastroesophageal pressure gradient on one hand and the presence of hiatal hernia, esophageal acid exposure and the presence of esophagitis on the other hand in a large group of GERD patients.

Figure 2 is a schematic representation of the esophagogastric junction (EGJ) in a patient with HH, illustrating the putative mechanism through which BMI influences HH development as supported by our study.

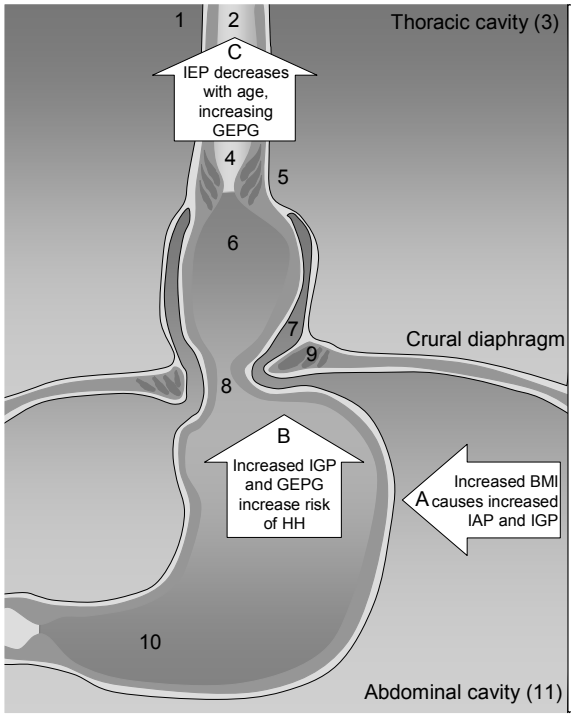


Figure 2.
Schematic representation of the esophago-gastric junction (EGJ) in a patient with a sliding hiatal hernia. The numbers denote the various structures, compartments and pressures influencing the transit of gastric contents across the EGJ. The elevated intra-abdominal pressure (IAP) in patients with high BMI (bold arrow A) increases intragastric pressure (IGP) (bold arrow B). Intraesophageal pressure (IEP) decreases with age (bold arrow C). These two factors contribute to increased gastroesopha-geal pressure gradient (GEPG) which in turn increases the risk of hiatal hernia.

1. Esophageal body: length, contractility and motility patterns
2. Intraesophageal pressure
3. Intrathoracic pressure
4. EGJ diameter
5. Lower esophageal sphincter: basal pressure and transient relaxations
6. Pressure in hiatal sac
7. Pressure in herniated peritoneal sac
8. Length of herniated portion of stomach
9. Crural diaphragm: muscle tone and hiatal diameter
10. Intragastric pressure
11. Intra-abdominal pressure

Our results confirm that IGP is associated with BMI during both inspiration and expiration, but only the inspiratory GEPG was predicted by BMI, because during expiration BMI predicted IEP with an effect size similar to the association with expiratory IGP. In addition a modest association between age and inspiratory IEP and GEPG was observed with a "mirror-image"-effect size, indicating that age influences the inspiratory GEPG only by

decreasing IEP. The importance of increased IGP and GEPG is illustrated by our finding that, in addition to the expected influence of BMI, both IGP and GEPG are strong, independent predictors of HH, with an increase of 1 kPa in IGP and GEPG causing an almost threefold increased risk of HH. No direct influence of BMI or mean IGP, IEP or GEPG on esophageal acid exposure was observed in this study. Only HH, age and esophageal motility performance predicted esophageal acid exposure during 24-hour pH-metry. Our results strongly suggest that BMI, IGP and GEPG can contribute to GERD pathogenesis by increasing the risk of developing HH.

The relationship between BMI, IGP and GEPG has been further clarified by the present study, and our findings fit with previous evidence in the literature. The fact remains, that the mechanism through which obesity increases IGP and the risk of developing GERD is still only partially understood. Intragastric pressure has been found to be approximately the same as intra-abdominal pressure, suggesting gastric wall tension does not contribute significantly to intragastric pressure[16]. Applying external pressure to the abdomen, as well as performing a Valsalva maneuver or raising one's legs, thereby increasing intra-abdominal pressure, causes an increase in intragastric pressure [17]. Furthermore, with a rise in intra-abdominal pressure, LES pressure is elevated accordingly and the LES moves to a more proximal position [17]. It is therefore conceivable that the BMI-associated increase in intragastric pressure that was found in the current study causes the LES to move more proximally as well. This could lead to a position of the LES above the crural diaphragm, causing a double-peaked pressure zone in the EGJ region. Indeed, Pandolfino et al. found that obesity is associated with increased spatial separation between the LES and the manometrically visualized crural diaphragm [11], a phenomenon that has been described as favoring gastroesophageal reflux by Bredenoord et al. [18]. This double-peaked pressure zone could be a prodrome of HH: Smith et al. investigated a group of eight power athletes with no radiographic evidence of HH in resting state, and found that seven could induce HH when they increased their intra-abdominal pressure by straining against a weight lifting belt [19]. The data from these studies, expanded with the findings from the present study, further clarify the mechanisms that could lead to the increased prevalence of HH [12, 13] and symptoms of GERD [7] in the obese.

Evidence from previous epidemiological studies points toward an increase in GERD prevalence with age (for an overview see Dent et al [1]). This is concordant with our finding that age is a predictor of inspiratory GEPG and esophageal acid exposure.

The findings that HH predicts supine and total acid exposure and that the risk of esophagitis increases fourfold with the presence of HH are a confirmation of results of earlier studies[20].

Patients with HH have a longer esophageal acid clearance time [21, 22] as well as a higher prevalence of pathological reflux [23]. It is also well established that acid clearance is affected by esophageal motility performance, as is confirmed by our finding that distal esophageal contraction amplitude predicts acid exposure time during 24-hour pH-metry. We could not confirm the relation between BMI and distal esophageal contraction amplitude that was found by Stacher et al. [24], however our findings do fit the evidence they provide.

The present study has some shortcomings. Firstly, only BMI was scored as a measure of obesity. Two recent reports suggest however that waist circumference (WC) is of greater importance than BMI in the increased risk of GERD in the obese [25, 26]. Secondly, in our study HH was diagnosed during endoscopy while fluoroscopy is the gold standard. Because of this, we might have misclassified some patients with HH as not having HH because during endoscopy a HH is not always visible. False positive diagnosis of HH however is implausible, which means that the effects we found could be underestimated by misclassification. HH size is important as well [20], but this also cannot be reliably determined during endoscopy as it may vary much from moment to moment, for this reason we did not take any data addressing HH size into account in the present study.

In conclusion, the present study provides further insight in the complex relationships between intragastric pressure, intraesophageal pressure, gastroesophageal pressure gradient, hiatal hernia, BMI and esophageal acid exposure in GERD patients. HH remains the most important determinant of esophageal acid exposure and esophagitis in GERD patients, but BMI, IGP and GEPG appear to be important in the development of HH.

References

1. Dent J, El Serag HB, Wallander MA, Johansson S. Epidemiology of gastrooesophageal reflux disease: A systematic review. Gut 2005; 54(5):710-717.

2. Holloway RH. The anti-reflux barrier and mechanisms of gastro-oesophageal reflux. Best Pract Res Clin Gastroenterol 2000; 14(5):681-699.

3. Van Herwaarden M, Samsom M, Smout A. Excess gastroesophageal reflux in patients with hiatus hernia is caused by mechanisms other than transient LES relaxations. Gastroenterology 2000; 119(6):1439-1446.

4. Scheffer RCH, Gooszen HG, Hebbard GS, Samsom M. The role of transsphincteric pressure and proximal gastric volume in acid reflux before and after fundoplication. Gastroenterology 2005; 129(6):1900-1909.

5. Pandolfino JE, Zhang QG, Ghosh SK, Han A, Boniquit C, Kahrilas PJ. Transient lower esophageal sphincter relaxations and reflux: mechanistic analysis using concurrent fluoroscopy and high-resolution manometry. Gastroenterology 2006; 131(6):1725-1733.

6. Bredenoord AJ, Weusten BLAM, Timmer R, Smout AJPM. Gastro-oesophageal reflux of liquids and gas during transient lower oesophageal sphincter relaxations. Neurogastroent Motil 2006; 18(10):888-893.

7. Jacobson BC, Somers SC, Fuchs CS, Kelly CP, Camargo CA. Body-mass index and symptoms of gastroesophageal reflux in women. N Engl J Med 2006; 354(22):2340-2348.

8. Piretta L, Alghisi F, Anzini F, Corazziari E. Prevalence of overweightedness in patients with gastro-esophageal reflux. World J Gastroenterol 2007; 13(34):4602-4605.

9. Hampel H, Abraham NS, El Serag HB. Meta-analysis: Obesity and the risk for gastroesophageal reflux disease and its complications. Ann Intern Med 2005; 143(3):199-211.

10. El Serag HB, Tran T, Richardson P, Ergun G. Anthropometric correlates of intragastric pressure. Scand J Gastroenterol 2006; 41(8):887-891.

11. Pandolfino JE, El Serag HB, Zhang Q, Shah N, Ghosh SK, Kahrilas PJ. Obesity: A challenge to esophagogastric junction integrity. Gastroenterology 2006; 130(3):639-649.

12. Stene-Larsen G, Weberg R, Froyshov Larsen I, Bjortuft O, Hoel B, Berstad A. Relationship of overweight to hiatus hernia and reflux oesophagitis. Scand J Gastroenterol 1988; 23(4):427-432.

13. Wilson LJ, Ma W, Hirschowitz BI. Association of obesity with hiatal hernia and esophagitis. Am J Gastroenterol 1999; 94(10):2840-2844.

14. Wu JC, Mui LM, Cheung CM, Chan Y, Sung JJ. Obesity is associated with increased transient lower esophageal sphincter relaxation. Gastroenterology 2007; 132(3):883-889.

15. Bredenoord AJ, Weusten BL, Roelofs JM, Smout AJ. The gastro-oesophageal pressure inversion point revisited. Scand J Gastroenterol 2003; 38(8):812-818.

16. De Waele J, Berrevoet F, Reyntjens K, Pletinckx P, De laet I, Hoste E. Semicontinuous intra-abdominal pressure measurement using an intragastric Compliance catheter. Intensive Care Med 2007; 33(7):1297-1300.

17. Dodds WJ, Hogan WJ, Miller WN, Stef JJ, Arndorfer RC, Lydon SB. Effect of increased intraabdominal pressure on lower esophageal sphincter pressure. Am J Dig Dis 1975; 20(4):298-308.

18. Bredenoord AJ, Weusten BL, Timmer R, Smout AJ. Intermittent spatial separation of diaphragm and lower esophageal sphincter favors acidic and weakly acidic reflux. Gastroenterology 2006; 130(2):334-340.

19. Smith ABD, Dickerman RDD, McGuire CSD, East JWD, McConathy WJP, Pearson HFD. Pressure-Overload-Induced Sliding Hiatal Hernia in Power Athletes. J Clin Gastroenterol 1999; 28(4):352-354.

20. Patti MG, Goldberg HI, Arcerito M, Bortolasi L, Tong J, Way LW. Hiatal hernia size affects lower esophageal sphincter function, esophageal acid exposure, and the degree of mucosal injury. Am J Surg 1996; 171(1):182-186.

21. Johnson LF, DeMeester TR, Haggitt RC. Esophageal epithelial response to gastroesophageal reflux. A quantitative study. Am J Dig Dis 1978; 23(6):498-509.

22. Emerenziani S, Habib FI, Ribolsi M et al. Effect of hiatal hernia on proximal oesophageal acid clearance in gastro-oesophageal reflux disease patients. Aliment Pharmacol Ther 2006; 23(6):751-757.

23. Xenos ES. The role of esophageal motility and hiatal hernia in esophageal exposure to acid. Surg Endosc 2002; 16(6):914-920.

24. Stacher G, Lenglinger J, Eisler M et al. Esophageal acid exposure in upright and recumbent postures: roles of lower esophageal sphincter, esophageal contractile and transport function, hiatal hernia, age, sex, and body mass. Dig Dis Sci 2006; 51(11):1896-1903.

25. Corley DA, Kubo A, Levin TR et al. Abdominal obesity and body mass index as risk factors for Barrett's esophagus. Gastroenterology 2007; 133(1):34-41.

26. El-Serag HB, Ergun GA, Pandolfino J, Fitzgerald S, Tran T, Kramer JR. Obesity increases oesophageal acid exposure. Gut 2007; 56(6):749-755.

Chapter 4.

Patients with physiological acid exposure and positive symptom association scores: a distinct group within the GERD spectrum

DR de Vries
MA van Herwaarden
AJPM Smout
M Samsom

Neurogastroenterology and motility, in press

ABSTRACT

Background: Studies comparing pH-metrically well-characterized GERD patients with physiological reflux to GERD patients with pathological reflux, with regard to clinical and epidemiological data, are lacking.

Methods: We included 273 GERD patients with pathological 24-hour pH-monitoring (pH+), defined as pH<4 ≥ 6% of time. A symptom index (SI) ≥ 50% was considered positive, as well as a symptom association probability (SAP) ≥ 95%. We included 84 GERD patients with physiological acid exposure (pH-) and a positive SI and/or SAP. Manometry and endoscopy reports were reviewed. Subjects completed questionnaires about demographics and medical history, FD and IBS, the Nepean Dyspepsia Index symptom score and the RAND-36 quality of life scale.

Results: pH- patients were younger (45 v. 50 years, P=.003), more often female (60% v. 39%, P=.001), smoked more (31% v. 19%, P=.021) and reported PPI failure more often (47% v 32%, P=.027). A hypotensive LES was less common in pH- patients (18% v 34%, P=.008) and distal esophageal contraction amplitude was higher (11 v 9.5 kPa, P=.045). pH- patients had hiatal hernia and esophagitis less often (48% v 73%, P<.0005; 36% v 54%, P=.012, respectively). pH- patients less often reported no other symptoms besides GERD (20% v 34%, P=.015). pH- patients scored worse at the Nepean (reflux 19 v 12 out of 39, P<.0005; dyspepsia 54 v 38 out of 156, P<.0005).

Conclusion: In the subgroup of patients who have physiological esophageal acid exposure the enhancement of the perceived symptom burden appears to be the most important mechanism in GERD pathogenesis.

INTRODUCTION

Gastro-esophageal reflux disease (GERD) ranks among the most common ailments in the Western world [1]. According to the Montreal definition, GERD is a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications [2]. In patients with classical symptoms of GERD, the diagnostic approach with the best supporting evidence is combined 24-hour pH/impedance monitoring with symptom association analysis, which is able to connect subjective symptoms to objective intra-esophageal events [3]. Different measurement outcomes classify patients into different groups, each with its own therapeutic approach. To a certain extent gastroesophageal reflux is physiological, however, in a minority of the population reflux episodes that amount to a physiological total acid exposure cause GERD nonetheless. These patients perceive physiological amounts of acid reflux as bothersome, and often their symptoms are a reason to seek a physician's care. Most patients with classical GERD symptoms indeed do have a positive 24-hour measurement outcome and can be treated with medical or surgical anti-reflux therapy. Patients with classical GERD symptoms but with a negative 24-hour measurement outcome who also do not respond to PPI treatment are diagnosed with functional heartburn, according to the Rome III criteria, and treated accordingly [4]. Patients in whom the 24-hour measurement shows that physiological amounts of acid reflux causes GERD symptoms, however, are treated differently than patients with pathological amounts of acid reflux. These patients are treated with medical therapy only, predominantly proton pump inhibition (PPI). In case of persisting symptoms, however, they generally are not considered candidates for anti-reflux surgery. In several studies, patients with GERD caused by physiological reflux are considered viscerally hypersensitive [5]. Despite the common nature of this problem, studies comparing patients with classical GERD symptoms with regard to demographics, objective findings (other than the pH-metry) or patient-reported outcomes are lacking. This study aimed to compare pH-metrically characterized GERD patients with physiological acid exposure to patients with pathological acid exposure with regard to the parameters mentioned above.

METHODS

Subjects

Patients were selected from the population referred to the University Medical Center Utrecht because of GERD symptoms or non-cardiac chest pain from 2004 to 2007. We included 357 patients in whom GERD was proven during 24-hour intra-esophageal pH-monitoring (24-hour combined pH-impedance monitoring was at that time not yet validated for clinical purposes) after a one-week wash-out period off PPI and other acid-suppressing medications. GERD was defined as having symptoms of retrosternal pain, heartburn and/or regurgitation at least weekly, and a total percentage of time with pH under 4 ≥ 6% of time and/or a positive SI (≥ 50%) [6] and/or SAP (≥ 95%) [7]. Analysis of the temporal association between reflux symptom episodes and reflux events was performed after atypical symptoms had been excluded from the analysis on the basis of the diaries kept by the patients. Patients with a history of surgery or organic disease affecting GI-motility or sensibility were excluded.

Patients were classified as having physiological reflux (pH-, N=84) if the total acid exposure time was less than 6% of the 24-hour monitoring period, or pathological reflux (pH+, N=273) if acid exposure was equal to or higher than 6% of this period. All patients with acid exposure <6% had positive SI and/or SAP.

Questionnaires

Sociodemographic questionnaire

All patients completed a questionnaire addressing sociodemographic data, medical history, and medication use. When patients used medication for reflux symptoms, they were prompted to report if these suppressed their symptoms or not.

Nepean Symptom Score

The symptom score from the Nepean Dyspepsia Index [8] was used to assess reflux symptoms and dyspeptic symptoms in the two weeks prior to questionnaire completion. This instrument encompasses symptom frequency, severity and bothersomeness, reflected by a summary score ranging from 0 to 13 points.

Reflux and Rome-II derived symptom score

All patients completed an 18-item questionnaire inquiring about symptoms in the year preceding the inclusion, addressing reflux symptoms including their diurnal occurrence, as well as symptoms consistent with the Rome-II criteria for FD and IBS [9].

Rand-36

All patients completed the validated Dutch translation of the 36-item SF-36 questionnaire [10-12]. This instrument measures general health-related quality of life (HRQoL), divided into nine domains. These domains are: physical functioning, social functioning, physical and emotional role limits (i.e. perceived limitation of daily activities caused by either physical or emotional problems), mental health, vitality, bodily pain, general health experience and health change. Each of these subscales has a score ranging from 0 –100, with a score of 100 representing unimpaired HRQoL.

Upper GI endoscopy

Upper GI endoscopy reports were retrieved from hospital records in our hospital or collected from referring hospitals with patients' consent. Hiatal hernia was defined as distance between esophagogastric junction and diaphragmatic impression of 3 cm or more. Esophagitis was scored according to the LA classification.

Statistical analysis

Categorical variables were tested using chi-square testing. Continuous variables were tested using Student's T-test.

RESULTS

Subject characteristics

Table 1 shows the characteristics of patients in the pH- and pH+ groups. The group of pH- patients was significantly younger, contained relatively more women and more tobacco users than the pH+ group. With regard to medical history, pH- patients more often reported to have visited a physician for epigastric or other abdominal complaints besides GERD symptoms. In concordance with Dutch medical consensus, patients used PPI as primary medical treatment of reflux symptoms, in a minority supplemented with H2-antagonists and/or antacids. Patients in the pH- group reported incomplete symptom relief by PPI treatment more often. pH- patients reported more GI symptoms in their family histories.

Table 1. Patient characteristics

	N	Sex F(%)	Age Mean (SEM)	BMI Mean (SEM)	Tobacco Use N(%)	Alcohol Daily N(%)	PPI failure N(%)*	History of only GERD	GI symptoms in family history
pH+	273	106(39)	50(0.81)	27(0.26)	52(19)	134(49)	54(32)	195(72)	58(21)
pH-	84	50(60)	45(1.45)	26(0.42)	26(31)	36(43)	31(47)	43(52)	32(38)
P-value		.001	.003	.064	.021	.318	.027	.001	.002

SEM: standard error of the mean; BMI: body mass index; PPI: proton pump inhibitor; GI:gastrointestinal; pH+: GERD patients with 24-hour esophageal acid exposure ≥ 6% of the time; pH-: GERD patients with 24-hour esophageal acid exposure < 6% of the time but with positive symptom association probability. *PPI effect was reported by 237 subjects, 171 pH+ and 66 pH-

Objective GERD parameters

Outcomes of esophageal manometry assay and upper GI endoscopy are displayed in table 2. Patients in the pH- group had better outcomes of esophageal manometry than patients in the pH+ group: a hypotensive lower esophageal sphincter was less common and the motor function of the esophageal body, as reflected by the distal esophageal contraction amplitude, was better. pH- patients had less often an HH and the prevalence and severity of esophagitis were lower as well.

Table 2. Objective GERD parameters

	Esophageal manometry				Upper gastrointestinal endoscopy			
	N	LES pressure Mean (SEM) kPa	hypotensive LES (<0.6kPa) N(%)	distal amplitude Mean (SEM) kPa	N	HH N(%)	esopha-gitis N(%)	severe esophagitis and/ or Barrett's N(%)
pH+	244	1.0 (0.05)	82 (34)	9.5 (0.37)	218	158 (72)	116 (54)	39 (18)
pH-	74	1.2 (0.09)	13 (18)	11.0 (0.70)	60	29 (48)	21 (36)	1 (2)
P-value		.158	.008	.045		<.0005	.012	.002

LES: lower esophageal sphincter; SEM: standard error of the mean; HH: hiatus hernia; pH+: GERD patients with 24-hour esophageal acid exposure ≥ 6% of the time; pH-: GERD patients with24-hour esophageal acid exposure < 6% of the time but with positive symptom index and/or symptom association probability; severe esophagitis: LA grade C or D.

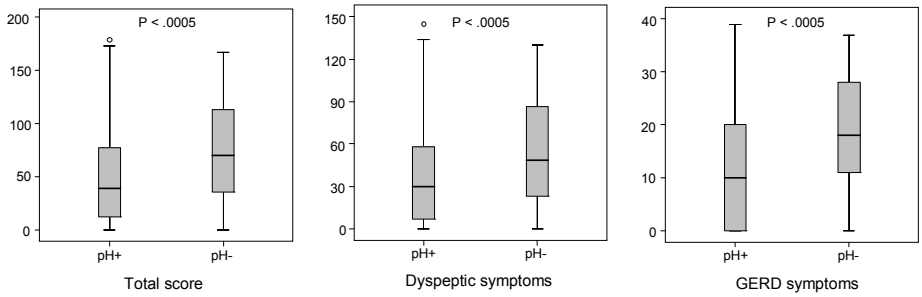
When the 24-hour pH reports were compared with regard to the predominant reflux pattern (upright, supine or both) no significant differences were seen. Also, when the symptom

registrations were compared, no significant differences in number of registered symptoms were observed, and no significant difference in the distribution of these symptoms during the 24 hour period was seen.

Subjective parameters

Figure 1 shows the symptom scores patients reported about the two weeks prior to the filling out of the questionnaires. The pH- group reported more and worse symptoms than the pH+ group (73 v 49 out of 195, P<.0005). When only GERD symptoms (regurgitation, retrosternal pain, heartburn) were taken into account, the average score in the pH- group was higher than in the pH+ group as well (19 v 12 out of 39, P<.0005), and the same was seen for the remaining (dyspeptic) symptoms (54 v 38 out of 156, P<.0005).

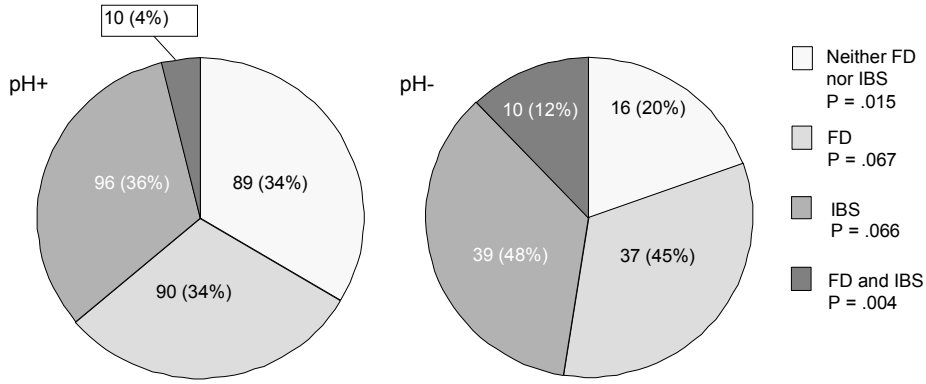
Figure 1. Box plots of outcomes of the Nepean symptom scores.



These three plots depict, from left to right, the total score, the score on questions regarding dyspeptic symptoms and the score regarding GERD symptoms. pH- patients scored more frequent, more severe and more bothersome symptoms in both domains.

Figure 2 shows the distribution of symptoms consistent with FD and IBS in the pH- and pH+ groups. Significantly more subjects in the pH- group reported symptoms that qualified both for FD and IBS, and significantly less pH- patients reported no concomitant FD or IBS but only GERD symptoms. In the group with only GERD symptoms 23% of subjects reported insufficient effect of PPIs, while in the group with concomitant FD and/or IBS, 41% reported non-response to PPIs (chi-square P=0.011).

Figure 2. Pie plots of the distribution of symptoms fulfilling criteria of FD and IBS in the pH- and pH+ groups.



Numbers are N(%). In the pH- group, fewer patients reported only symptoms consistent with GERD, FD and IBS were more prevalent in this group.

The pH- group reported more symptoms consistent with FD and more symptoms consistent with IBS, these differences both showed trends towards significance. The prevalence of reported nocturnal reflux symptoms was not different between the two groups.

Health-Related Quality of Life

The pH- group reported lower HRQoL on three out of nine subscales of the RAND-36 questionnaire: social functioning, physical role limits and pain. The first scale reflects the limitation experienced by a subject in fulfilling social tasks, the second reflects the limitations in completing daily tasks caused by physical complaints and the last scale reflects limitations in daily life experienced as a result of physical pain.

DISCUSSION

The current study compares a large group of GERD patients with physiological reflux but with positive symptom association to GERD patients with pathological reflux. These two groups of patients have been treated as separate groups in a large portion of the literature [13], our results provide evidence to justify this separation. The main results are that patients with physiological reflux are younger and more often female, that they respond to PPI treatment less well, that they perceive their symptoms as being more bothersome, that they have worse health-related quality of life in several domains and that they have more concomitant symptoms of the GI tract than patients with pathological reflux.

Previous studies involving visceral hypersensitivity in GERD have focused mainly on non-erosive reflux disease (NERD). There is a considerable overlap between the pH- group and the NERD group; 64% of the pH- patients did not have esophagitis and are therefore NERD patients. NERD patients have been shown to have symptoms of FD and IBS more often, and to have a higher sensitivity to acid perfusion, especially in the proximal esophagus, which is enhanced by the presence of gas in the refluxate [14-18]. NERD patients do not have esophagitis but they do have abnormal pH-metry outcomes in most definitions, which makes these patients very interesting indeed. However, the NERD/ERD division has two major drawbacks when visceral hypersensitivity is concerned. Firstly, NERD patients with pathological amounts of acid reflux are mixed with NERD patients with a positive symptom-reflux association score as the only abnormal pH-metry outcome. Secondly, patients with esophagitis, regardless of their symptoms, are all grouped together as having erosive reflux disease. However, lots of subjects with esophagitis are symptom-free [19]. Therefore, it is difficult to prove or disprove theories about visceral hypersensitivity in GERD when GERD patients are divided in NERD and ERD patients. The division between pH- and pH+ patients, regardless of the presence of esophagitis, ensures that the current study involves an undisputedly viscerally hypersensitive group of GERD patients.

This having been said, the severity of reflux in a 24-hour period can vary widely in one patient, the concordance between repeated measurements not being higher than 77% [20]. In this light, one could argue that the patients with physiological reflux included in the current study could have been classified as such merely by coincidence, and that at any other day, chance would have a different subgroup of patients selected. Our results strongly counter this argument, however, with the outcomes of both manometry and endoscopy. During manometry, the patients with physiological reflux performed better: they had better esophageal motility as reflected by distal contraction amplitude, and hypotensive LES was

seen less often. During endoscopy, esophagitis and hiatus hernia were seen less frequently, and the damage to the esophageal lining was less severe. These objective findings corroborate the classification of these patients as having physiological reflux. Moreover, a recent report showed that the SAP, by which the patients in the current study were qualified as GERD patients notwithstanding the physiological amount of reflux they had during 24-hour pH-metry, is a highly reproducible parameter [21].

One can assume that primary care physicians started PPI therapy in all symptomatic patients regardless of the outcome of pH monitoring that was to come, since this is standard practice in the Netherlands [22]. It can therefore be assumed that acid suppressant use was evenly distributed among the pH- and pH+ groups at the time of endoscopy, thus rendering PPI use insignificant as a confounder for esophagitis in the current study.

The current study provides evidence for a distinction in clinical practice between GERD patients with and without pathological esophageal acid exposure. Since non-response to PPI therapy appears to be associated with the presence of concomitant FD and/or IBS, physicians should actively address FD and IBS in patients complaining about insufficient effects of acid suppressing medication.

Prospective study designs are needed to determine the predictive value of patient-reported outcome measures. Such a design would also provide information about patients with negative pH study outcomes who do not respond to PPI treatment, i.e. functional heartburn patients. The importance of 24-hour pH-monitoring including symptom association analysis remains. Studies on drugs targeting hypersensitivity of the esophagus, e.g. low-dose antidepressants [23], are needed to provide alternative treatment strategies for this complex patient group. Also of note, therapies with coping and acceptance as a goal have been proven effective in IBS [24, 25], a syndrome that is characterized by hypersensitivity of the GI tract [26, 27], much like GERD with physiological acid exposure. Therefore, for GERD patients with physiological reflux, as a supplement to PPI therapy clinicians should consider early referral to coping and acceptance therapy, and consider off-label prescription of hypersensitivity altering drugs.

References

1. Dent J, El Serag HB, Wallander MA, Johansson S. Epidemiology of gastrooesophageal reflux disease: A systematic review. Gut 2005; 54(5):710-717.

2. Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. Am J Gastroenterol 2006; 101(8):1900-1920.

3. Bredenoord AJ, Weusten BLAM, Curvers WL, Timmer R, Smout AJPM. Determinants of perception of heartburn and regurgitation. Gut 2006; 55(3):313-318.

4. Drossman DA. The functional gastrointestinal disorders and the Rome III process. Gastroenterology 2006; 130(5):1377-1390.

5. Fass R, Tougas G. Functional heartburn: the stimulus, the pain, and the brain. Gut 2002; 51(6):885-892.

6. Wiener GJ, Richter JE, Copper JB, Wu WC, Castell DO. The Symptom Index - A Clinically Important Parameter of Ambulatory 24-Hour Esophageal Ph Monitoring. Am J Gastroenterol 1988; 83(4):358-361.

7. Weusten BL, Roelofs JM, Akkermans LM, Berge-Henegouwen GP, Smout AJ. The symptom-association probability: an improved method for symptom analysis of 24-hour esophageal pH data. Gastroenterology 1994; 107(6):1741-1745.

8. Talley, Haque, Wyeth et al. Development of a new dyspepsia impact scale: the Nepean Dyspepsia Index. Alimentary Pharmacology and Therapeutics 1999; 13(2):225-235.

9. Drossman DA. The functional gastrointestinal disorders and the Rome II process. Gut 1999; 45:1-5.

10. Hunt SM, Mckenna SP. Validating the Sf-36. Brit Med J 1992; 305(6854):645.

11. Mant JWF, Jenkinson C, Murphy MFG, Clipsham K, Marshall P, Vessey MP. Use of the Short Form-36 to detect the influence of upper gastrointestinal disease on self-reported health status. Qual Life Res 1998; 7(3):221-226.

12. Ware JE, Sherbourne CD. The Mos 36-Item Short-Form Health Survey (Sf-36) .1. Conceptual-Framework and Item Selection. Med Care 1992; 30(6):473-483.

13. 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.

14. Neumann H, Monkemuller K, Kandulski A, Malfertheiner P. Dyspepsia and IBS symptoms in patients with NERD, ERD and Barrett's esophagus. Dig Dis 2008; 26(3):243-247.

15. Wu JC, Cheung CM, Wong VW, Sung JJ. Distinct clinical characteristics between patients with nonerosive reflux disease and those with reflux esophagitis. Clin Gastroenterol Hepatol 2007; 5(6):690-695.

16. Zimmerman J, Hershcovici T. Bowel symptoms in nonerosive gastroesophageal reflux disease: nature, prevalence, and relation to acid reflux. J Clin Gastroenterol 2008; 42(3):261-265.

17. Emerenziani S, Sifrim D, Habib FI et al. Presence of gas in the refluxate enhances reflux perception in non-erosive patients with physiological acid exposure of the oesophagus. Gut 2008; 57(4):443-447.

18. Emerenziani S, Ribolsi M, Sifrim D, Blondeau K, Cicala M. Regional oesophageal sensitivity to acid and weakly acidic reflux in patients with non-erosive reflux disease. Neurogastroenterol Motil 2008.

19. Ronkainen J, Aro P, Storskrubb T 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):275-285.

20. Johnsson F, Joelsson B. Reproducibility of ambulatory oesophageal pH monitoring. Gut 1988; 29(7):886-889.

21. Aanen MC, Bredenoord AJ, Numans ME, Samsom M, Smout AJ. Reproducibility of Symptom Association Analysis in Ambulatory Reflux Monitoring. The American Journal of Gastroenterology 2008; in press.

22. Numans ME, De Wit NJ, Dirven JAM et al. NHG-standaard maagklachten (tweede herziening). Huisarts en Wetenschap 2003; 46:690-700.

23. 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):365-370.

24. Robinson A, Lee V, Kennedy A et al. A randomised controlled trial of self-help interventions in patients with a primary care diagnosis of irritable bowel syndrome. Gut 2006; 55(5):643-648.

25. Wilson S, Maddison T, Roberts L, Greenfield S, Singh S. Systematic review: the effectiveness of hypnotherapy in the management of irritable bowel syndrome. Aliment Pharmacol Ther 2006; 24(5):769-780.

26. Costantini M, Sturniolo GC, Zaninotto G et al. Altered esophageal pain threshold in irritable bowel syndrome. Dig Dis Sci 1993; 38(2):206-212.

27. Trimble KC, Farouk R, Pryde A, Douglas S, Heading RC. Heightened visceral sensation in functional gastrointestinal disease is not site-specific. Evidence for a generalized disorder of gut sensitivity. Dig Dis Sci 1995; 40(8):1607-1613.

Chapter 5.

Treatment received by patients with gastroesophageal reflux disease is not associated with CYP2C19 genotype status

DR de Vries
MA van Herwaarden
M Samsom

submitted

ABSTRACT

Background: Acid suppression with proton pump inhibitors (PPIs) is the treatment of first choice for gastroesophageal reflux disease (GERD). Incomplete symptom resolution during medical treatment is not uncommon however, and can lead to invasive treatment, e.g. endoscopic or surgical anti-reflux therapy. Insufficient acid suppression by PPIs can contribute to incomplete symptom relief. Most proton pump inhibitors are metabolized by Cytochrome P450 (CYP) 2C19. In Caucasians inter-individual variability in acid-inhibitory effect of PPIs is mainly caused by CYP2C19*2 genotype. The effect of CYP2C19*2 genotype on the treatment received by patients with GERD is unknown.

Methods: We included 259 GERD patients, defined by 24-hour pH-metry. They were divided into three groups based on the received treatment: 92 received medical treatment, 39 endoscopic anti-reflux therapy and 128 underwent anti-reflux surgery; the latter groups together formed the group that received invasive, as opposed to medical, treatment. A structured telephone survey was conducted among surgically treated patients assessing the reasons they underwent surgery. CYP2C19*2 was genotyped using molecular beacons.

Results: The main reason to undergo anti-reflux surgery was persistent heartburn during PPI treatment. However, CYP2C19*2 genotype distribution was similar in all groups and did not differ from frequencies reported in healthy Dutch Caucasian volunteers.

Conclusions: CYP2C19*2 genotype is similar in groups of GERD patients that have received medical treatment, endoscopic anti-reflux therapy or anti-reflux surgery. This suggests that the influence of CYP2C19*2 genotype on the acid-inhibitory effect of PPIs is not reflected by the treatment GERD patients receive.

INTRODUCTION

Gastro-esophageal reflux disease (GERD) is a common disorder: approximately 20% of the Caucasian population suffers from reflux symptoms at least weekly [1]. Proton pump inhibitors (PPIs) are the most potent medical therapy for GERD. Patients are primarily medically treated, insufficient symptom relief on maximum dose PPI however can be an indication for invasive treatment, i.e. endoscopic anti-reflux therapy or surgical fundoplication [2].

PPIs are mainly metabolized in the liver by cytochrome P450 (CYP) 2C19. CYP2C19 polymorphism is the main cause of inter-individual variability of the gastric acid-inhibitory effects of PPIs [3]. Several variant alleles of the CYP2C19 gene associated with an inactive enzyme have been detected. CYP2C19*2 is by far the most frequent defective variant in Caucasians [4]. Based on the occurrence of defective alleles patients are classified into homozygous extensive metabolizers (homozygous-EM (wt/wt)), heterozygous extensive metabolizers (heterozygous-EM (wt/*2)) and poor metabolizers (PM (*2/*2)). The distribution of homozygous-EM, heterozygous-EM and PM genotypes shows wide interethnic differences. In healthy Dutch Caucasians the frequency of the *2 allele has been reported to be 13.3% [5]. The rapid metabolism of PPIs in homozygous-EM is reflected by the highest incidence of nocturnal gastric acid breakthrough and the lowest cure rate of esophagitis [3, 6]. It is conceivable that the CYP2C19 genotype status affects the success of PPI treatment in patients with GERD. One could therefore hypothesize that invasive treatment is more often observed among homozygous-EM.

MATERIALS AND METHODS

Subjects

Two hundred and fifty-nine Caucasian GERD patients participated in this study. All patients had symptoms of heartburn, regurgitation or non-cardiac chest pain. In all patients GERD diagnosis had been established by 24-hour esophageal pH recording, and defined by the presence of pathological reflux (% time with pH<4 ≥ 6) and/or symptom index (SI) ≥ 50% and/or symptom association probability (SAP) ≥ 95% [7, 8]. Patients with severe comorbidity or paraesophageal hernia were excluded, thus minimizing bias in the indication for invasive therapy. 24-hour esophageal pH recording was performed according to our standard protocol [7].

Based on the treatment they had received, patients were classified into three groups. One group consisted of 92 patients who were on standard medical treatment. The second group consisted of 39 patients who underwent endoscopic anti-reflux therapy (ESD (endoscopic suturing device, Wilson-Cook Medical, Winston-Salem, NC, USA), EndoCinch (C.B. Bard, Inc., Murray Hill, NJ, USA) or EsophyX (EsophyX, Inc., Redmond, WA, USA)). The third group consisted of 128 patients who underwent antireflux surgery (Nissen fundoplication or Belsey Mark-IV).

CYP2C19*2 genotyping

Genomic DNA was extracted from whole blood using the QIAamp DNA blood minikit (Qiagen, Hilden, Germany). Genotyping was performed by Molecular Beacon assay using the iCycler iQ real-time PCR detection system (BioRad, Hercules, CA, USA). The assay was carried out in a total volume of 25 µl, containing 50 ng of genomic DNA, 12.5 µl 2× iQ Supermix (BioRad, Hercules, CA, USA), 500 nM of each primer, and 200 nM of each molecular beacon. $MgCl_2$ was added to obtain a final concentration of 4 mM. The following primers were used: 5'-GAGCTTGGCATATTGTATCTATACC-3' (forward) and 5'-TACTTTC-TCCAAAATATCACTTTCC-3' (reverse). Sequences of the Molecular Beacons were 5'-FAM-CGCGATTTATGGGTTCCCGGGAAATAATCATCGCG-DABCYL-3' (wt-allele specific) and 5'-TXR-CGCGATTTATGGGTTCCTGGGAAATAATCATCGCG-DABCYL-3' (*2-allele specific). The PCR thermal cycling protocol applied consisted of an initial denaturation and enzyme activation step of 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 45 s. In each run representative samples from each genotype were inserted. To validate genotyping by the Molecular Beacon assay, polymerase chain reaction-based restriction fragment length polymorphism analysis was performed in a set of randomly chosen patients. For this purpose the polymerase chain reaction fragments were digested with *Sma*I overnight at 25°C and separated by 2.5% agarose gel electrophoresis. *Sma*I cuts the wt allele in two parts (107 and 64 bp). Concordance was 100%.

Telephone survey

A representative sample of patients (N=49) who had undergone anti-reflux surgery was subjected to a telephone survey inquiring about the main reason, in the patients' opinion, to undergo fundoplication. The answer was classified as one of the following: 1) persistent heartburn during maximum dose PPIs, 2) persistent regurgitation during maximum dose PPIs, 3) unwillingness to take lifetime medication, 4) side effects of PPIs, 5) other reasons.

After the patients had spontaneously stated the main reason, they were asked in detail about the presence or absence of any of these five problems.

Statistical analysis

Sex, age and BMI were compared with chi-square tests and anova. Concordance with Hardy-Weinberg equilibrium was calculated using an online tool [9]. Allele frequencies were compared using chi-square testing. Influence of genotype on received treatment was analyzed with a logistic regression model adjusting for sex, age and BMI.

Medical Ethics Committee

All patients signed informed consent forms after the protocol was approved by the medical ethics committee at the University Medical Center Utrecht, in accordance with the declaration of Helsinki.

RESULTS

Patient characteristics, genotype distribution and allele frequencies are displayed in table 1.

Table 1. Patient characteristics and genotype distribution

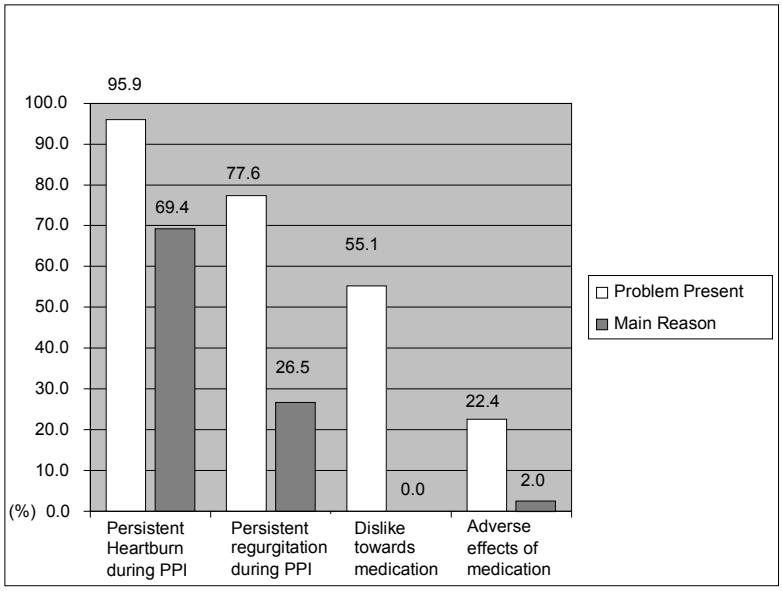
	N	Males (%)	Age mean	BMI mean	Genotypes (N(%)) wt/wt	wt/*2	*2/*2	allele frequency wt	*2
Total	259	147 (57)	47.6	26.9	199 (76.8)	56 (21.6)	4 (1.5)	.876	.124
Medical	92	49 (53.3)	50.0	26.6	66 (71.7)	25 (27.2)	1 (1.1)	.853	.147
Endoscopic	39	21 (53.8)	45.7	26.2	31 (79.5)	7 (17.9)	1 (2.6)	.885	.115
Surgical	128	77 (60.2)	46.6	27.2	102 (79.7)	24 (18.8)	2 (1.6)	.891	.109

wt/wt: homozygous extensive metabolizers, wt/*2: heterozygous extensive metabolizers, *2/*2: poor metabolizers.

No significant difference in gender distribution, age and BMI was found between the treatment groups. The majority of the patients were homozygous-EM in all three groups, approximately 20% were heterozygous-EM and some patients were PM. The CYP2C19*2 genotype status was not significantly different between the three patient groups or between

medically treated patients and invasively treated patients combined. Furthermore, neither the total group (i.e. referred GERD patients) nor any of the three patient subgroups differed from the allele frequency found in healthy Dutch volunteers [5].

Figure 1. Patients' reasons to undergo anti-reflux surgery



Most patients had multiple reasons to undergo an anti-reflux operation. However, persistent heartburn and in lesser extent persistent regurgitation during high dose PPIs appeared to be the main underlying reasons.

Figure 1. shows the percentage of patients in whom persistent heartburn and regurgitation during PPIs, reluctance to take lifetime medication and side effects on PPIs played a role in the decision to undergo the fundoplication. Most patients confirmed more than one problem during PPI treatment. Note, however, that 69.4% of the patients stated persistent heartburn on PPIs as the main reason to undergo anti-reflux surgery. Moreover, 95.9% confirmed the presence of persistent heartburn during PPI treatment in the time before surgery had been performed.

DISCUSSION

Although GERD is a common disorder, with a prevalence of 10-20% in the general population in the Western world [1], only a minority undergoes endoscopic anti-reflux therapy or anti-reflux surgery because of refractory GERD. CYP2C19*2 genotype is the main determinant of the acid inhibitory effect of PPIs in Caucasians. In this study we established for the first time the CYP2C19*2 status in a large group of patients with proven GERD. As expected the genotype distribution did not deviate from that in the general population, because CYP2C19 polymorphism does not play a role in the pathogenesis of GERD but may affect treatment.

This study reveals that the presence of the defective allele is not associated with a lower odds of invasive therapy. Post hoc analysis indicated that the statistical power was 0.87 (observed α 0.125, observed R^2 0.041, 4 predictors (genotype, age, sex and BMI)) [10].

Several studies showed remarkable effects of CYP2C19 polymorphism on intragastric acidity and the occurrence of nocturnal acid breakthrough during PPI treatment [3, 6]. Moreover, Furuta et al. and Kawamura et al. reported higher cure rates of erosive esophagitis in PM and heterozygous-EM compared to homozygous-EM after an 8-week treatment period with lansoprazole 30 mg daily [6, 11]. Based on these data we expected an increased frequency of homozygous-EM in GERD patients who received invasive therapy. In agreement with data of healthy Caucasians, approximately 77% of our GERD patients were homozygous-EM. However, in contrast with our hypothesis we observed frequencies of homozygous-EM in the group of patients treated with invasive anti-reflux therapies similar to those in the group on medical treatment. Egan et al. reported that gastric acid suppression, but not esophageal acid exposure was affected by the presence of CYP2C19 variant alleles [12]. This counters our hypothesis to some extent, however Egan's study included only 60 patients, receiving a wide variety of types and doses of PPIs, rendering the power to support this negative finding substantially compromised. Still, esophageal acid exposure and efficiency of gastric acid suppression may be poorly correlated. Furthermore, one could argue that our study design lacks a pre-operative pH-metry during PPI treatment, however, complete symptom resolution while on PPI therapy does not preclude pathological esophageal acid exposure [13]. Efficiency of acid suppression is not the main determinant of GERD symptom relief. Other factors, such as coping behavior and esophageal hypersensitivity, which are both left untouched by PPIs, contribute to GERD symptoms as well. In conclusion, this study showed invasive treatment, as opposed to medical treatment, received by GERD patients is not associated with the CYP2C19*2 genotype status.

References

1. Dent J, El Serag HB, Wallander MA, Johansson S. Epidemiology of gastrooesophageal reflux disease: A systematic review. Gut 2005; 54(5):710-717.

2. Palmer K. Review article: indications for anti-reflux surgery and endoscopic anti-reflux procedures. Aliment Pharmacol Ther 2004; 20(s8):32-35.

3. Sagar M, Tybring G, Dahl ML, Bertilsson L, Seensalu R. Effects of omeprazole on intragastric pH and plasma gastrin are dependent on the CYP2C19 polymorphism. Gastroenterology 2000; 119(3):670-676.

4. Wedlund PJ. The CYP2C19 enzyme polymorphism. Pharmacology 2000; 61(3):174-183.

5. Tamminga W, Wemer J, Oosterhuis B, Zeeuw Rd, Leij Ld, Jonkman J. The prevalence of CYP2D6 and CYP2C19 genotypes in a population of healthy Dutch volunteers. European Journal of Clinical Pharmacology 2001; V57(10):717-722.

6. Kawamura M, Ohara S, Koike T et al. The effects of lansoprazole on erosive reflux oesophagitis are influenced by CYP2C19 polymorphism. Aliment Pharmacol Ther 2003; 17(7):965-973.

7. Weusten BL, Roelofs JM, Akkermans LM, Berge-Henegouwen GP, Smout AJ. The symptom-association probability: an improved method for symptom analysis of 24-hour esophageal pH data. Gastroenterology 1994; 107(6):1741-1745.

8. Wiener GJ, Richter JE, Copper JB, Wu WC, Castell DO. The Symptom Index - A Clinically Important Parameter of Ambulatory 24-Hour Esophageal Ph Monitoring. Am J Gastroenterol 1988; 83(4):358-361.

9. www.kursus.kvl.dk/shares/vetgen/_Popgen/genetik/applets/kitest.htm. 1999.

10. www.danielsoper.com/statcalc/calc09.aspx. 2007.

11. Furuta T, Shirai N, Watanabe F et al. Effect of cytochrome P4502C19 genotypic differences on cure rates for gastroesophageal reflux disease by lansoprazole. Clin Pharmacol Ther 2002; 72(4):453-460.

12. Egan LJ, Myhre GM, Mays DC, Dierkhising RA, Kammer PP, Murray JA. CYP2C19 pharmacogenetics in the clinical use of proton-pump inhibitors for gastro-oesophageal reflux disease: variant alleles predict gastric acid suppression, but not oesophageal acid exposure or reflux symptoms. Alimentary Pharmacology & Therapeutics 2003; 17(12):1521-1528.

13. Gerson LB, Boparai V, Ullah N, Triadafilopoulos G. Oesophageal and gastric pH profiles in patients with gastro-oesophageal reflux disease and Barrett's oesophagus treated with proton pump inhibitors. Aliment Pharmacol Ther 2004; 20(6):637-643.

Chapter 6.

Gastroesophageal reflux disease is associated with the C825T polymorphism in the G-protein β3 subunit gene (GNB3)

DR de Vries
JJM Ter Linde
MA van Herwaarden
AJPM Smout
M Samsom

American Journal of Gastroenterology 2009; 104:281–285

ABSTRACT

Background: Visceral hypersensitivity plays a role in the etiology of reflux symptoms. Familial clustering and twin studies demonstrated a genetic predisposition to GERD. G-protein coupled receptors (GPCR) mediate the response to acid, neurotransmitters and humoral factors modulating esophageal sensory function. Thus, polymorphisms in G-proteins are putative genetic factors contributing to GERD manifestation. A functional polymorphism in the G-protein β3 subunit gene (GNB3) is associated with functional dyspepsia, in which visceral hypersensitivity is implicated in symptom generation.

We evaluated the association of the GNB3 C825T polymorphism with GERD and GERD subgroups classified according to esophageal acid exposure time, symptom-reflux correlation or co-existence of FD and/or IBS symptoms.

Methods: In total 363 GERD patients, defined as esophageal pH<4 ≥6% of time and/or SI ≥ 50% or SAP ≥ 95%, participated. In addition, 373 healthy controls free of gastrointestinal symptoms were studied. Genotyping was performed by molecular beacon assay.

Results: The CT genotype was more prevalent in GERD patients relative to healthy controls (adjusted odds ratio (OR) = 1.43, 95% CI 1.04-1.98). GERD patients sensitive to physiological amounts of reflux displayed a higher OR (1.59), as did GERD patients with a positive symptom association score (1.50). The strongest association was detected in patients without concomitant FD and/or IBS symptoms (OR = 1.66).

Conclusion: GERD is associated with GNB3 C825T. The results for GERD subgroups support the hypothesis that enhanced perception of reflux events as a consequence of the increased signal transduction upon GPCR activation associated with the 825T allele underlies this association.

INTRODUCTION

Gastroesophageal reflux disease (GERD) is characterized by mucosal injury or inflammation of the esophagus and/or symptoms occurring as a consequence of reflux of gastric contents into the esophagus. The typical symptoms are heartburn and regurgitation, and, less specifically, retrosternal pain. These symptoms are very common in the Western society with 10-20% of the population suffering from one or more of the typical symptoms at least once a week [1-3].

In GERD patients there exists a wide range of symptom severity and frequency relative to the magnitude of esophageal acid exposure [4-6]. These observations suggest interindividual variability in esophageal perception. Patients with esophageal acid exposure within physiological range and positive symptom-reflux correlation respond to esophageal balloon distension at lower thresholds than healthy subjects [7]. Furthermore, hyperalgesia to esophageal acid perfusion and electrical stimulation of the esophagus has been demonstrated in GERD [8, 9]. These studies indicate that visceral hypersensitivity plays a role in the etiology of reflux symptoms. Heightened perception may be caused by an exaggerated sensitivity of esophageal extrinsic primary afferent nerve terminals and/or distorted processing and representation of gut signals in the brain[10, 11]. Central visceral afferent pathway hypersensitivity has been demonstrated in GERD patients as well [9]. Furthermore, a considerable overlap exists between GERD and functional gastrointestinal symptom complexes like functional dyspepsia (FD) and irritable bowel syndrome (IBS)[12]. The presence of symptoms apparently originating from multiple gastrointestinal regions also indicates that altered processing of visceral stimuli within the CNS plays a role in GERD symptom generation.

Epidemiological data justify theory formation about a genetic component in the pathophysiology of GERD. It has been shown that reflux symptoms tend to cluster in families[13, 14], and twin studies document a higher risk of GERD in monozygotic relative to dizygotic twins[15, 16]. Genetic factors may contribute to interindividual variability in response to esophageal acid exposure. These genetic factors may be related to receptors involved in the transmission of gastroesophageal sensations, or transporters and enzymes determining the availability of neurotransmitters and humoral factors. Furthermore, it can be anticipated that an underlying second messenger abnormality affects the response to acid and to the release of neurotransmitters and humoral factors modulating gastroesophageal sensory function. Putative second messenger candidates include the heterotrimeric G-proteins. The distribution of G-protein coupled receptors (GPCRs) at multiple levels within the brain-gut

axis make polymorphisms in G-proteins attractive candidates to pursue. The 825T allele of the functional polymorphism in the G protein β3 subunit gene (GNB3) is associated with an increased intracellular signal transduction [17]. Moreover, this genetic polymorphism was found to be associated with functional dyspepsia, another upper GI disorder in which heightened visceral perception is thought to play a role [18-20].

In this study we evaluated the association of the GNB3 C825T polymorphism with GERD and subgroups of GERD patients classified according to esophageal acid exposure time, symptom-reflux correlation or co-existence of FD and/or IBS symptoms.

METHODS

Subjects

Three hundred and sixty-three unrelated GERD patients with chronic heartburn, regurgitation or non-cardiac chest pain participated in this study. Patients were prospectively selected from the referrals to our hospital between 2004 and 2008. GERD diagnosis was established using 24-hour esophageal pH recording [21] off PPI. GERD was defined as esophageal pH below 4 for ≥ 6% of the time , and/or a positive symptom association score for heartburn or regurgitation (i.e. SI ≥ 50%[22] or SAP ≥ 95%[23]). Patients reported symptoms and meals on diary cards, thus atypical symptoms and time during meals could be excluded from the measurement period. Patients were excluded if they had any history of disease and/or surgery that affected gastrointestinal motility or gastric acid secretion at the time of the pH monitoring. All patients were of Western European descent.

Three hundred and seventy three unrelated healthy volunteers, all of Western European descent, were recruited by public advertisement concurrently with the patient recruitment, in two approximately three-week sessions. They were selected after a structured telephone survey, which inquired about medical history and general health, gastrointestinal complaints and medication use, and after completing a questionnaire (specified below) had not yielded any symptoms.

The study was approved by the medical ethics committee of the University Medical Center Utrecht, and written informed consent was obtained from all participants.

Assessment of gastrointestinal symptoms

The patients and volunteers filled out a questionnaire that assessed symptoms of GERD and of FD and IBS according to the Rome II criteria [24]. Based on this questionnaire GERD

patients were divided into patients with only symptoms of GERD, or with concomitant symptoms of FD, IBS or both.

Genotyping

Genomic DNA was extracted from whole blood using the QIAamp DNA blood minikit (Qiagen, Hilden, Germany). Genotyping was performed by Molecular Beacon assay using the iCycler iQ real-time PCR detection system (BioRad, Hercules, CA, USA). The assay was carried out in a total volume of 25 µl, containing 50 ng of genomic DNA, 12.5 µl 2× iQ Supermix (BioRad, Hercules, CA, USA), 1000 nM of the forward primer, 250 nM of the reverse primer, 200 nM of the FAM-labeled beacon, and 400 nM of the TXR-labelled beacon. $MgCl_2$ was added to obtain a final concentration of 5 mM. The following primers were used: 5'-TGCCGCTTGTTTGACCTG-3' (forward) and 5'-CAGTTGAAGTCGTCGTAG-CC-3' (reverse). Sequences of the Molecular Beacons were 5'-FAM-CGGCTCGAAGGCCA-CGGACGTGATGGAGCCG-DABCYL-3' (C-allele specific) and 5'-TXR-CGGCTCAGAAGG-CCACAGACGTGATGGAGCCG-DABCYL-3' (T-allele specific). The PCR thermal cycling protocol applied consisted of an initial denaturation and enzyme activation step of 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 63°C for 1 min and 72°C for 45 s. In each run representative samples from each genotype were inserted. To validate genotyping by the Molecular Beacon assay, PCR-based restriction fragment length polymorphism analysis was performed in a set of randomly chosen subjects. For this purpose the PCR fragments were digested with *BsaJ*I overnight at 60°C and separated by 2.5% agarose gel electrophoresis. The C allele yielded DNA fragments of 77 and 57 bp, whereas the T allele PCR product remained uncut. Concordance was 100%.

Data analysis

Genetic associations were tested with chi-square tests. The genotype distribution in patients and controls was tested for Hardy-Weinberg equilibrium using chi square test incorporated in an online tool (www.kursus.kvl.dk/shares/vetgen/_Popgen/genetik/applets/kitest.htm). (The Hardy-Weinberg equilibrium is a range of predicted genotype frequencies in a group of subjects, given the prevalence of the different alleles of a polymorphic gene. It signifies that a control population has been selected in which none of the possible genotypes is over- or underrepresented. Patient populations can diverge from the equilibrium as a results of polymorphism effects.)

Binary logistic regression analysis with patient/control as response variable was performed to calculate adjusted odds ratios with 95 percent confidence intervals. The investigated SNP

has been associated with obesity [25], therefore BMI was added to the regression model to adjust for confounding.

Statistical power

Sample size calculations were performed as described by Zar [26], assuming a frequency of the T-allele in the control group of 0.30 [27]. We considered an OR of 1.6 for assessing the necessary sample size, and calculated that against the 373 available controls 270 patients would be sufficient for detection.

RESULTS

Subject characteristics and GNB3 C825T genotype distribution are summarized in table 1.

Table 1. Subject characteristics and GNB3 C825T genotype distribution

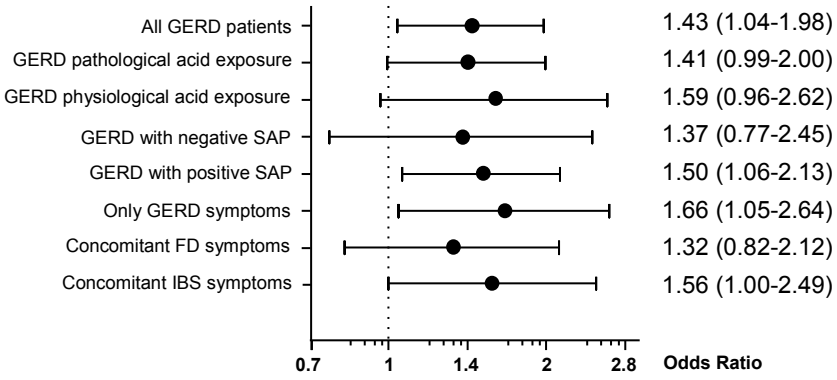
	Subject characteristics			Allele distribution			
Group	N (male)	Age mean (SD)	BMI mean (SD)	CC N (%)	CT N (%)	TT N (%)	HW
Healthy controls	373 (120)	40.1 (12.3)	24.1 (3.9)	199 (53.4)	138 (37.0)	36 (9.7)	yes
All GERD patients	363 (205)	48.6 (13.6)	26.7 (4.1)	167 (46.0)	171 (47.1)	25 (6.9)	no
GERD pathol. acid exposure	279 (171)	49.7 (13.6)	26.9 (4.2)	129 (46.2)	129 (46.2)	21 (7.5)	yes
GERD physiol. acid exposure	84 (34)	45.0 (13.0)	26.0 (3.8)	38 (45.2)	42 (50.0)	4 (4.8)	yes
GERD with negative SAP	64 (34)	51.8 (15.5)	26.9 (4.9)	30 (46.9)	28 (43.8)	6 (9.4)	yes
GERD with positive SAP	271 (155)	47.7 (12.9)	26.7 (3.8)	122 (45.0)	131 (48.3)	18 (6.6)	no
Only GERD symptoms	106 (76)	48.2 (14.1)	26.4 (3.6)	47 (44.3)	54 (50.9)	5 (4.7)	no
Concomitant FD symptoms	128 (62)	49.7 (12.3)	26.6 (4.0)	60 (46.9)	56 (43.8)	12 (9.4)	yes
Concomitant IBS symptoms	136 (66)	47.5 (13.7)	27.0 (4.7)	60 (44.1)	68 (50.0)	8 (5.9)	no

GERD pathol. acid exposure: patients with pathological esophageal acid exposure during 24-hour pH-monitoring, i.e. ≥ 6% of time with esophageal pH <4. GERD physiol. acid exposure: patients with physiological acid exposure during 24-hour pH-monitoring, i.e. < 6% of time with esophageal pH <4, but with positive SI and/or SAP. Only GERD symptoms: patients with GERD without symptoms of FD or/and IBS. Concomitant FD/IBS symptoms: GERD patients with concomitant symptoms of FD or IBS, respectively. When added these groups contain more than the total 363 patients, this is due to overlap between FD and IBS: 20 patients had both symptoms of FD and of IBS. HW: Hardy-Weinberg equilibrium, i.e. Hardy-Weinberg $\chi^2 < 3.84$.

The control group contained more female subjects than the patient group (P<.0005) and had a lower BMI and age (both P<.0005). The genotype distribution of GNB3 C825T in the controls was similar to that previously reported for Western European populations [27], and did not deviate from Hardy-Weinberg equilibrium.

The genotype distribution in the GERD patients was not in Hardy-Weinberg equilibrium (χ^2 4.58). The CT genotype was more prevalent (P=0.005) in GERD patients relative to healthy controls, while the frequencies of both homozygous genotypes were reduced. Only for the CC genotype this reduction was significantly different (P=0.046). An adjusted odds ratio of 1.43 (95% CI 1.04-1.98) for GERD versus healthy was calculated for heterozygotes relative to CC homozygotes (Figure 1).

Figure 1. Adjusted odds ratios for GERD patients or subgroups of GERD patients



GERD pathological acid exposure: patients with pathological esophageal acid exposure during 24-hour pH-monitoring, i.e. ≥ 6% of time with esophageal pH <4. GERD physiological acid exposure: patients with physiological acid exposure during 24-hour pH-monitoring, i.e. < 6% of time with esophageal pH <4, but with positive SI and/or SAP. Only GERD symptoms: patients with GERD without symptoms of FD or/and IBS. Concomitant FD/IBS symptoms: GERD patients with concomitant symptoms of FD or IBS, respectively.

When the patients were divided according to esophageal acid exposure time, a higher odds ratio with a trend towards significance (1.59 (95% CI 0.96-2.62)) was observed for the group of patients with physiological acid exposure (i.e. <6% total time with pH below four during 24-hour pH-metry).

When the patients were classified based on symptom-reflux correlation, an increased OR was detected for patients with a positive symptom association score (OR 1.50 (95% CI 1.06-2.13)). In contrast, no association between the genotype of the GNB3 C825T polymorphism and GERD patients with a negative symptom association score was observed.
The strongest OR was found in the group of GERD patients with no symptoms consistent with either FD or IBS: OR 1.66 (95% CI 1.05-2.64). In the group with concomitant symptoms consistent with FD, there was no association with the CT genotype. In the group with concomitant symptoms consistent with IBS there was an association with the CT genotype (OR 1.56 (95% CI 1.00-2.49).

DISCUSSION

The genetic factors contributing to the manifestation of GERD are largely unknown. We identified an association between the heterozygous genotype of the GNB3 C825T polymorphism and GERD. The association was stronger in patients sensitive to physiological amounts of reflux. Moreover, the association was absent in patients with pathological acid exposure but negative symptom-reflux correlation. Therefore, we hypothesize that enhanced perception of reflux events as a consequence of the increased signal transduction upon GPCR activation associated with the GNB3 825T allele is the mechanism underlying this association.
The genetic factor identified may affect the sensory and pain threshold of esophageal extrinsic primary afferent nerve terminals and/or central processing of visceral stimuli from the esophagus. It is known that the activation threshold of transient receptor potential VI (TRPV1), one of the acid-sensitive ion channels expressed on esophageal extrinsic primary afferent nerve terminals, is lowered through protein kinase-mediated signaling pathways induced by some GPCRs [28]. These GPCRs are activated by mediators released in injured and inflamed tissue. Thus, in carriers of the GNB3 825T allele with esophagitis the response to protons of TRPV1 and the subsequent signal processing may be augmented, thereby increasing sensory transmission of reflux events to the CNS. Moreover, the enhanced production of IL-8 in the esophageal mucosa of even patients with endoscopy negative reflux disease [29], suggests that inflammatory substances are increased in all GERD patients. These can activate protease-activated, bradykinin, and prostaglandin receptors, all excitatory GPCRs on esophageal extrinsic primary afferent terminals. In addition this may lead to heterologous sensitization of TRPV1 in patients with endoscopy negative reflux

disease as well. Activation of nociceptive circuits within the dorsal horn of the spinal cord is the result of extrinsic afferent nerves releasing several neurotransmitters, including substance P and calcitonin gene related peptide. Receptors for these two neurotransmitters belong to the GPCR family [30, 31]. It is conceivable that in the presence of the GNB3 825T allele the gain of sensory transmission at the spinal level through these receptors is increased. In the brain stem cholecystokinin (CCK) and corticotropin-releasing factor (CRF) are involved in the modulation of visceral sensitivity. Both CCK and CRF exert their effects through excitatory GPCRs [32, 33]. In this way the higher prevalence of GNB3 C825T heterozygotes may contribute to enhanced central processing of esophageal stimuli in GERD patients. Furthermore, the effect of the GNB3 825T allele may be manifested via receptors for neurotensin, which have been implicated in spinal descending facilitatory pathways [34].

The association was absent in SAP- patients. These patients are not hypersensitive, and the majority of them probably had been referred because of other GERD-related problems than symptoms, such as refractory esophagitis or Barrett's epithelium. This finding supports the theory that the association is related to visceral hypersensitivity.

There was no association found in the subgroup of patients with concomitant FD symptoms. The genotype distribution, however, did resemble those found in the two studied populations described in the paper by Holtmann et al. where in the FD groups the CC genotype was more prevalent than the CT genotype [19]. The subgroup with concomitant FD in the present study suffers from an overlap syndrome of upper GI symptoms qualifying both as GERD and as FD, and the different nature of the association of GNB3 C825T with both diseases could explain the absence of association in this subgroup. In a study by Andresen et al. no association of the GNB3 C825T with IBS was found [27]. This could explain the fact that in the subgroup with IBS, the association with GERD remains standing.

The size of the study population could be construed as a limitation. Power was sufficient to demonstrate the association in the entire group of GERD patients, however, the subgroups that were appointed are relatively small. Therefore, conclusions drawn from these sub-analyses should be interpreted with care. However, the subgroup analyses provide valuable clues to help interpret the nature of the association, since all point towards enhanced visceral sensitivity. Perhaps, future joined efforts to create larger databases could consolidate the findings of the present study.

Our control group was no population sample but consisted of a selection of individuals who were thoroughly checked for the absence of any gastrointestinal symptoms. Moreover, the controls had no serious medical history, including any of the multifactorial diseases that the

investigated SNP has been associated with thus far [35]. This explains the slightly lower frequency of the T-allele in our control group compared to the total number of Western Europeans investigated to date (0.28 v. 0.30, chi-square $P<0.05$) [27].

In conclusion, we found an association of GERD with the GNB3 C825T polymorphism. In subgroups of GERD patients for whom the esophageal sensitivity to acid reflux was the foremost disease characteristic, the association was stronger. This result emphasizes the importance of visceral hypersensitivity, besides reflux mechanisms, in the occurrence of GERD symptoms.

References

1. Dent J, El Serag HB, Wallander MA, Johansson S. Epidemiology of gastrooesophageal reflux disease: A systematic review. Gut 2005; 54(5):710-717.

2. Locke GR, Zinsmeister AR, Fett SL, Melton LJ, Talley NJ. Overlap of gastrointestinal symptom complexes in a US community. Neurogastroent Motil 2005; 17(1):29-34.

3. Louis E, DeLooze D, Deprez P et al. Heartburn in Belgium: prevalence, impact on daily life, and utilization of medical resources. Eur J Gastroenterol Hepatol 2002; 14(3):279-284.

4. Bredenoord AJ, Weusten BLAM, Curvers WL, Timmer R, Smout AJPM. Determinants of perception of heartburn and regurgitation. Gut 2006; 55(3):313-318.

5. Cicala M, Emerenziani S, Caviglia R et al. Intra-oesophageal distribution and perception of acid reflux in patients with non-erosive gastro-oesophageal reflux disease. Aliment Pharmacol Ther 2003; 18(6):605-613.

6. Weusten B, Akkermans L, vanBerge-Henegouwen G, Smout A. Symptom perception in gastroesophageal reflux disease is dependent on spatiotemporal reflux characteristics. Gastroenterology 1995; 108(6):1739-1744.

7. 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.

8. Mehta AJ, De Caestecker JS, Camm AJ, Northfield TC. Sensitization to painful distention and abnormal sensory perception in the esophagus. Gastroenterology 1995; 108(2):311-319.

9. Sarkar S, Aziz Q, Woolf CJ, Hobson AR, Thompson DG. Contribution of central sensitisation to the development of non-cardiac chest pain. Lancet 2000; 356(9236):1154-1159.

10. Fass R, Naliboff B, Higa L et al. Differential effect of long-term esophageal acid exposure on mechanosensitivity and chemosensitivity in humans. Gastroenterology 1998; 115(6):1363-1373.

11. Mayer EA, Gebhart GF. Basic and clinical aspects of visceral hyperalgesia. Gastroenterology 1994; 107(1):271-293.

12. De Vries DR, Van Herwaarden MA, Baron A, Smout A, Samsom M. Concomitant functional dyspepsia and irritable bowel syndrome decrease health-related quality of life in gastroesophageal reflux disease. Scand J Gastroenterol 2007; 42(8):951-956.

13. Romero Y, Cameron AJ, Locke GR, III et al. Familial aggregation of gastroesophageal reflux in patients with Barrett's esophagus and esophageal adenocarcinoma. Gastroenterology 1997; 113(5):1449-1456.

14. Trudgill NJ, Kapur KC, Riley SA. Familial clustering of reflux symptoms. Am J Gastroenterol 1999; 94(5):1172-1178.

15. Cameron AJ, Lagergren J, Henriksson C, Nyren O, Locke GR, III, Pedersen NL. Gastroesophageal reflux disease in monozygotic and dizygotic twins. Gastroenterology 2002; 122(1):55-59.

16. Mohammed I, Cherkas LF, Riley SA, Spector TT, Trudgill NJ. Genetic influences in gastro-oesophageal reflux disease: a twin study. Gut 2003; 52(8):1085-1089.

17. Siffert W, Rosskopf D, Siffert G et al. Association of a human G-protein beta3 subunit variant with hypertension. Nat Genet 1998; 18(1):45-48.

18. Camilleri CE, Carlson PJ, Camilleri M et al. A study of candidate genotypes associated with dyspepsia in a U.S. community. Am J Gastroenterol 2006; 101(3):581-592.

19. Holtmann G, Siffert W, Haag S et al. G-protein beta 3 subunit 825 CC genotype is associated with unexplained (functioncal) dyspepsia. Gastroenterology 2004; 126(4):971-979.

20. Lelyveld Nv, Ter Linde JJM, Schipper M, Samsom M. Candidate Genes in Functional Dyspepsia. Neurogastroenterol Motil 2008;(in press):in press.

21. Van Herwaarden M, Samsom M, Smout A. Excess gastroesophageal reflux in patients with hiatus hernia is caused by mechanisms other than transient LES relaxations. Gastroenterology 2000; 119(6):1439-1446.

22. Wiener GJ, Richter JE, Copper JB, Wu WC, Castell DO. The Symptom Index - A Clinically Important Parameter of Ambulatory 24-Hour Esophageal Ph Monitoring. Am J Gastroenterol 1988; 83(4):358-361.

23. Weusten BL, Roelofs JM, Akkermans LM, Berge-Henegouwen GP, Smout AJ. The symptom-association probability: an improved method for symptom analysis of 24-hour esophageal pH data. Gastroenterology 1994; 107(6):1741-1745.

24. Drossman DA. The functional gastrointestinal disorders and the Rome II process. Gut 1999; 45:1-5.

25. Siffert W, Forster P, Jockel KH et al. Worldwide ethnic distribution of the G protein beta 3 subunit 825T allele and its association with obesity in Caucasian, Chinese, and Black African individuals. Journal of the American Society of Nephrology 1999; 10(9):1921-1930.

26. Zar JH. Biostatistical analysis, 4th edition, Prentice Hall International, Inc, New Jersey. Biostatistical analysis. New Jersey: Prentice Hall International, Inc.; 1999.

27. Andresen V, Camilleri M, Kim HJ et al. Is there an association between GN beta 3-C825T genotype and lower functional gastrointestinal disorders? Gastroenterology 2006; 130(7):1985-1994.

28. Zhang H, Cang CL, Kawasaki Y et al. Neurokinin-1 receptor enhances TRPV1 activity in primary sensory neurons via PKCepsilon: a novel pathway for heat hyperalgesia. J Neurosci 2007; 27(44):12067-12077.

29. Yoshida N, Uchiyama K, Kuroda M et al. Interleukin-8 expression in the esophageal mucosa of patients with gastroesophageal reflux disease. Scand J Gastroenterol 2004; 39(9):816-822.

30. Adwanikar H, Ji G, Li W, Doods H, Willis WD, Neugebauer V. Spinal CGRP1 receptors contribute to supraspinally organized pain behavior and pain-related sensitization of amygdala neurons. Pain 2007; 132(1-2):53-66.

31. Greco R, Tassorelli C, Sandrini G, Di BP, Buscone S, Nappi G. Role of calcitonin gene-related peptide and substance P in different models of pain. Cephalalgia 2008; 28(2):114-126.

32. Ji G, Neugebauer V. Pro- and anti-nociceptive effects of corticotropin-releasing factor (CRF) in central amygdala neurons are mediated through different receptors. J Neurophysiol 2008.

33. Kovelowski CJ, Ossipov MH, Sun H, Lai J, Malan TP, Porreca F. Supraspinal cholecystokinin may drive tonic descending facilitation mechanisms to maintain neuropathic pain in the rat. Pain 2000; 87(3):265-273.

34. Neubert MJ, Kincaid W, Heinricher MM. Nociceptive facilitating neurons in the rostral ventromedial medulla. Pain 2004; 110(1-2):158-165.

35. Siffert W. G protein polymorphisms inhypertension, atherosclerosis, and diabetes. Annual Review of Medicine 2005; 56:17-28.

Chapter 7.

Serotonergic gene polymorphisms in the pathophysiology of gastroesophageal reflux disease

DR de Vries
JJM Ter Linde
MA van Herwaarden
AJPM Smout
M Samsom

submitted

ABSTRACT

Background: Twin studies and familial clustering studies point towards a hereditary susceptibility for gastroesophageal reflux disease (GERD). Modulation of serotonin (5-HT) signalling influences esophageal perception of acid reflux, lower esophageal sphincter (LES) pressure and peristaltic contraction amplitude and frequency. Functional genetic polymorphisms in the 5-HT signaling pathway are therefore valid candidate genes for associations with GERD.

Methods: 363 GERD patients who had undergone 24-hour intraesophageal pH monitoring and 371 healthy controls were genotyped for the 44bp insertion/deletion polymorphism in the promoter of the 5-HT transporter (SERT-P), and single nucleotide polymorphisms (SNPs) in the 5-HT_3-receptor A subunit (HTR3A 178 C/T) and tryptophan hydroxylase 2 (TPH2 -703 G/T). Genotype frequencies in patients and subgroups of patients were compared to healthy controls using binary logistic regression analysis.

Results: HTR3A 178T carriership combined with the SERT-P LL genotype was associated with GERD characterized by pathological reflux (odds ratio 1.7, 95% confidence interval 1.0 - 3.0), and GERD characterized by a hypotensive LES (relative to normotensive LES: odds ratio 2.2, 95% confidence interval 1.2 – 4.1; relative to healthy controls: odds ratio 3.4, 95% confidence interval 1.7 – 6.5). No single polymorphism was associated with GERD.

Conclusion: GERD is associatied with the combined SERT-P LL and HTR3A 178 CT/TT genotype, which results in lower 5-HT availability and more homopentameric 5-HT3 receptors consisting of only A subunits, which have lower affinity and desensitize more rapidly. The finding that the association was found in GERD patients with pathological reflux and stronger in GERD patients with hypotensive LES points towards a modulatory effect on LES-pressure and esophageal peristalsis.

INTRODUCTION

Gastroesophageal reflux disease (GERD) is characterized by mucosal injury or inflammation of the esophagus and/or symptoms occurring as a consequence of reflux of gastric contents into the esophagus[1]. The typical symptoms are heartburn and regurgitation, and, less specifically, retrosternal pain. These symptoms are very common in the Western society with 10-20% of the population suffering from one or more of the typical symptoms at least once a week [2].

Transient relaxations of the lower esophageal sphincter (LES), low LES resting pressure, as well as low distal contraction amplitudes have proven to be very important mechanisms in the pathophysiology of GERD [3]. The mechanisms underlying enhanced acid perception and symptom generation, however, have not been fully clarified yet. There is a poor correlation between symptom severity and frequency and the magnitude of esophageal acid exposure [4-6], suggesting interindividual variability in esophageal perception. Several studies indicate that visceral hypersensitivity plays a role in the etiology of reflux symptoms. Hyperalgesia to esophageal acid perfusion and electrical stimulation of the esophagus have been demonstrated in GERD [7, 8], as well as hypersensitivity to balloon distension [9]. Furthermore, a considerable overlap exists between GERD and functional gastrointestinal symptom complexes like functional dyspepsia (FD) and irritable bowel syndrome (IBS) [10]. The presence of symptoms apparently originating from multiple gastrointestinal regions also indicates that altered processing of visceral stimuli plays a role in GERD symptom generation.

Epidemiological data justify theory formation about a genetic component in the pathophysiology of GERD. It has been shown that reflux symptoms tend to cluster in families [11, 12], and twin studies document a higher risk of GERD in monozygotic relative to dizygotic twins [13, 14]. Genetic factors may contribute to esophageal motor abnormalities and interindividual variability in response to esophageal acid exposure.

Visceral sensitivity of the esophagus has been shown to be modulated by selective serotonin reuptake inhibitor (SSRI) citalopram [15]. Esophageal motility can be modulated by serotonergic agents as well: in two studies sumatriptan, a 5-HT1 agonist, prevented the decay of high-rate postprandial transient LES relaxations, it increased LES pressure and it increased the amplitude of esophageal contractions [16, 17]. In another study a partial 5-HT_3-agonist reduced reflux events [18]. 5-HT_4-agonists have been shown to alter esophageal peristalsis as well [19]. Because of both sensitivity- and motility-modulating effects on the

esophagus, serotonergic signaling pathways provide valid candidate genes for association studies in GERD.

SSRIs like citalopram are inhibitors of the serotonin transporter protein, or SERT. A functional 44 base pair insertion-deletion polymorphism has been described in the promoter region of the SERT gene (SERT-P) [20]. The short allele results in attenuated transcription of the SERT gene. The ensuing smaller amount of SERT causes less reuptake of serotonin and a higher availability of serotonin to 5-HT receptors. Another candidate gene polymorphism in serotonergic signaling is the functional single nucleotide polymorphism (SNP) in an upstream open reading frame (uORF) of the 5-HT 3 receptor subunit A gene (HTR3A 178 C/T) [21]. The T allele results in more transcript in vitro. Two pentameric forms of the 5-HT 3 receptor have been characterized one consisting of five A subunits and the other consisting of two B and three A subunits [22]. In SERT knockout mice more homopentameric receptors consisting of only 5-HT 3_A subunits were found, and this in turn resulted in lower affinity and more rapid desensitization of serotonergic enteric neurons [23]. These results suggest that higher prevalence of 5-HT 3 receptors consisting only of A subunits results in less effective signaling by serotonin. The functionality of the HTR3A 178C/T SNP has been further supported by studies of the central nervous system[24, 25]. Carriers of the T allele can be assumed to have lower affinity for serotonin and lower activity in various serotonergic signaling pathways in which 5-HT 3 receptors are involved.

A third candidate polymorphism resides in the neural variant of the tryptophan hydroxylase gene (TPH2) which catalyzes the rate-limiting step in serotonin synthesis. The -703G/T SNP has been associated with psychological traits and psychiatric disorders which makes this SNP a possibly helpful clue in theory formation of the pathophysiology of the role of serotonergic signaling in visceral hypersensitivity in GERD [26-29].

METHODS

Subjects

Patients

363 unrelated GERD patients with chronic heartburn, regurgitation or non-cardiac chest pain participated in this study. GERD diagnosis was established with 24-hour esophageal pH recording off proton pump inhibitors, preceded by esophageal manometry as previously described [30]. GERD was defined as esophageal pH below 4 for ≥ 6% of the time , and/or a positive symptom association score for heartburn or regurgitation (i.e. SI ≥ 50%[31] or SAP ≥ 95%[32]). Patients were excluded if they had any history of disease and/or surgery at the time of the pH monitoring that affected gastrointestinal motility or gastric acid secretion. All patients were of Western European descent.

Healthy controls

371 healthy volunteers, all of Western European descent, were recruited by public advertisement. They were selected after a structured interview, which inquired about medical history and general health, gastrointestinal complaints and medication use.

The study was approved by the medical ethics committee of the University Medical Center Utrecht, and written informed consent was obtained from all participants.

Genotyping

Genomic DNA was extracted from 200 µl whole blood using the QIAamp DNA blood minikit according to manufacturer's instructions (Qiagen, Hilden, Germany).

The SERT-P and the HTR3A 178 C/T (rs1062613) polymorphisms genotypes were genotyped as previously described by Van Lelyveld et al. [33].

TPH2 G-703T (rs4570625)

Genotyping was performed by Molecular Beacon[34] assay using the iCycler iQ real-time PCR detection system (BioRad, Hercules, CA, USA), with one Texas Red® (TXR) and one 6-carboxyfluorescein (FAM) labelled beacon. The assay was carried out in a total volume of 25 µl, containing 50 ng of genomic DNA, 12.5 µl 2× iQ Supermix (BioRad, Hercules, CA, USA), 1000 nM of the forward primer, 250 nM of the reverse primer, 200 nM of the FAM-labeled beacon, and 400 nM of the TXR-labeled beacon. $MgCl_2$ was added to obtain a final concentration of 5 mM. The primer sequences for the genotyping assay were as follows: forward primer GTGGCTAAATTGAACCCTTACC, reverse primer ACTCATTGACCAACTC-CATTTTATG. The sequence of the molecular beacons was as follows: 5'-FAM-CGCGTCA-

CTTGACATATT**C**TAATTTTGTGCATGCGACGCG-DABCYL-3' and 5'-TXR- CGCGTCACT-TGACATATT**A**TAATTTTGTGCATGCGACGCG-DABCYL-3'.

The PCR thermal cycling protocol applied consisted of an initial denaturation and enzyme activation step of 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 63°C for 1 min and 72°C for 45 s. In each run representative samples from each genotype were inserted. To validate genotyping by the Molecular Beacon assay, PCR-based restriction fragment length polymorphism analysis was performed in a set of randomly chosen subjects. For this purpose the PCR fragments were digested with *PsiI* overnight at 37°C and separated by 2.5% agarose gel electrophoresis. The T allele yielded two DNA fragments, whereas the G allele PCR product remained uncut. Concordance was 100%.

Statistical analysis and power calculation

Concordance with Hardy-Weinberg equilibrium was tested using an online tool (http://www.oege.org/software/hardy-weinberg.shtml). Prevalence of genotypes was compared between groups using chi-square tests. Odds ratios were calculated using binary logistic regression analysis correcting for age, sex and BMI. Hosmer-Lemeshow goodness of fit tests were performed for the regression models as measure of model fit and statistical soundness. Power calculations were performed as described by Zar [35]. Using the frequencies of the least common alleles in the control group (frequencies of short SERT promoter 0.42, HTR3A 178T 0.19 and TPH2 -703T 0.22, respectively) it was determined that the included number of subjects was sufficient to detect associations with odds ratios of ≥1.5 for SERT-P, ≥1.7 for HTR3A 178 C/T and ≥1.6 for TPH2 -703 G/T with 80% power and a significance level of <0.05.

Table 1. Genotype distributions in controls and GERD patients.

	N (% M)	Age (mean)	SERT-P (LL / LS / SS)	HTR3A 178C/T (CC/CT/TT)	TPH2 -703G/T (GG/GT/TT)	SERT-P LL and HTR3ACT/TT
Controls	371 (32)	40	122 / 187 / 62 33 / 50 / 17%	242 / 116 / 13 65 / 31 / 4%	226 / 131 / 14 61 / 35 / 4%	41 (11%)
All GERD Patients	363 (56)	49	129 / 174 / 60 36 / 48 / 17%	225 / 122 / 16 62 / 34 / 4%	212 / 131 / 20 58 / 36 / 6%	54 (15%)
Pathological Reflux	279 (61)	50	108 / 128 / 43 39 / 46 / 15%	176 / 91 / 12 63 / 33 / 4%	163 / 101 / 15 58 / 36 / 6%	44 (16%)
Physiol. Reflux, SI/SAP+	84 (42)	45	21 / 46 / 17 25 / 55 / 20%	49 / 31 / 4 58 / 37 / 5%	49 / 30 / 5 58 / 36 / 6%	10 (12%)
Normotensive LES	227 (58)	48	76 / 112 / 39 33 / 49 / 17%	145 / 73 / 9 64 / 32 / 4%	137 / 77 / 13 60 / 34 / 6%	28 (12%)
Hypotensive LES	97 (62)	49	37 / 42 / 18 38 / 43 / 19%	54 / 36 / 7 56 / 37 / 7%	56 / 36 / 5 58 / 37 / 5%	23 (24%)

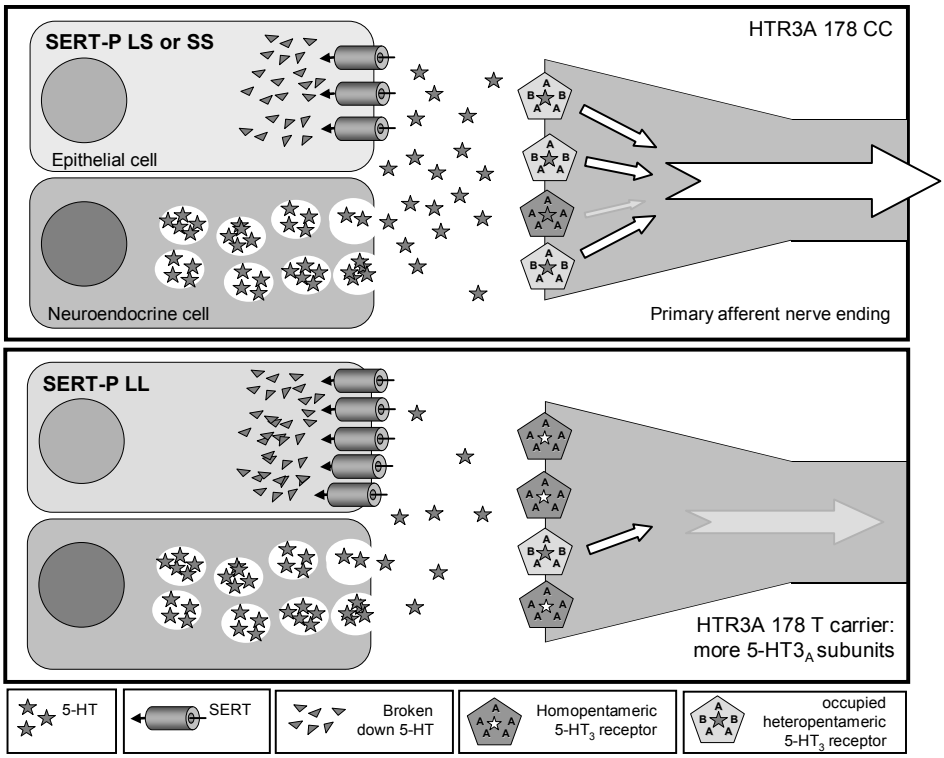
Genotype distributions for SERT-P, HTR3A 178 C/T and TPH2 -703 G/T are shown as determined in controls, GERD patients and subgroups. Esophageal manometry reports were available for 324 GERD patients. **Pathological reflux**: GERD patients with percentage of total time with pH <4 during 24-hour intraesophageal pH monitoring ≥6%. **Physiol. Reflux,SI/ SAP+**: GERD patients with percentage of total time with pH <4 during 24-hour intraesophageal pH monitoring <6%, but SI ≥50% and/or SAP ≥95%. **Normotensive LES**: GERD patients with resting LES pressure ≥ 0.6 kPa. **Hypotensive LES**: GERD patients with resting LES pressure < 0.6 kPa.
The rightmost column shows the number and percentage of HTR3A 178T carriers among subjects with the SERT-P LL genotype in each subgroup.

RESULTS

Subject characteristics and genotypes are displayed in table 1.

All genotype distributions fulfilled Hardy-Weinberg equilibrium in patients as well as in controls. The genotype distribution in controls was similar to previously reported figures in Western European populations [21, 36, 37]. No significantly higher or lower prevalence of the short or long SERT promoter, or one of the alleles of the HTR3A 178C/T polymorphism or the TPH2 -703G/T polymorphism was observed GERD patients compared to controls. In the subgroups of GERD patients with physiological and pathological acid exposure and in the subgroups with a normotensive or a hypotensive LES, no significant differences were observed either.

Figure 1. Schematic representation of the phenotype SERT-P LL and HTR3A 178 CT or TT



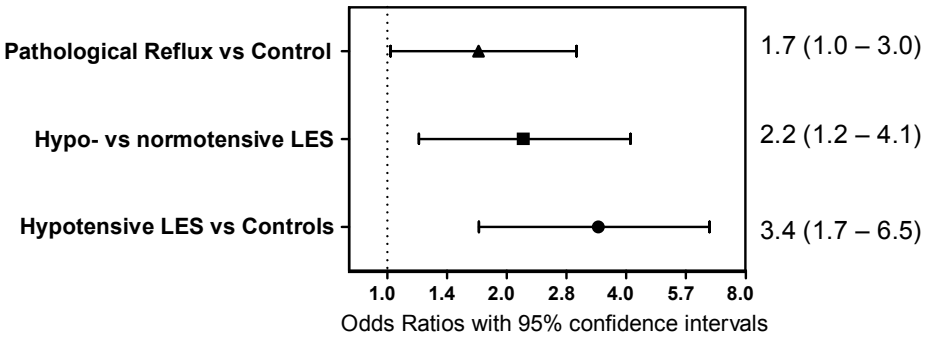
Top box: situation in subject with SERT-P LS or SS and HTR3A 178 CC genotype. Bottom box: in the SERT-P LL genotype more 5-HT transporters are present on epithelial cells, lowering 5-HT availability. In HTR3A 178 T carriers, more A subunits are synthesized, resulting in more homopentameric 5-HT_3 receptors with lower affinity for 5-HT and more rapid desensitization. This genotype was associated with GERD characterized by pathological reflux and with GERD characterized by a hypotensive LES.

The SERT-P LL genotype combined with either HTR3A 178 CT or TT can be assumed to have an additive effect on 5-HT3 receptor mediated signaling. The combined phenotype in which less 5-HT is available to a higher proportion of 5-HT3 receptors composed of A subunits, results in a decreased response to 5-HT. A schematic rendering of this genotype-phenotype effect is shown in figure 1.

No significantly higher prevalence of this combined genotype was seen in the GERD patient group as a whole compared to controls. However, as shown in figure 2, this genotype was more prevalent in the group of GERD patients with pathological acid exposure. Using binary

logistic regression analysis correcting for age, sex and BMI an odds ratio of 1.7 (95% confidence interval 1.0 – 3.0) was calculated. In patients with a hypotensive LES, the prevalence of combined SERT LL genotype with HTR3A 178T carriership was more than twice as high as in controls (24% v. 11%, P=0.001). Binary logistic regression analysis yielded an adjusted odds ratio of 3.4 (95% confidence interval 1.7 – 6.5) for this association. When GERD patients with a hypotensive LES were compared to patients with a normotensive LES, a higher prevalence of SERT-P LL combined with HTR3A 178T carriership was found as well. Binary logistic regression analysis yielded an odds ratio of 2.2 (95% confidence interval 1.2 – 4.1) for this effect.

Figure 2. HTR3A 178T carriership in subjects with the SERT-P LL genotype – associations with GERD, GERD with pathological reflux and GERD with hypotensive LES



Odds ratios calculated with binary logistic regression analysis, adjusted for age, sex and BMI. Pathological reflux: GERD patients with percentage of total time with pH <4 during 24-hour intraesophageal pH monitoring ≥6%. . Hypotensive LES: GERD patients with resting LES pressure < 0.6 kPa. Normotensive LES: GERD patients with resting LES pressure ≥ 0.6 kPa.

When patients with defective distal esophageal contractions (mean contraction amplitude < 8.5 kPa) were analyzed separately no significant associations were found (data not shown). Combining the SERT-P and HTR3A 178 C/T genotypes with TPH2 -703 G/T genotypes did not bring to light any other associations (data not shown).

DISCUSSION

The present study is the first to investigate functional gene polymorphisms of the serotonergic signaling pathway in a large group of GERD patients. The main findings are that the individual polymorphisms are not associated with GERD, however, we found an association with the combination of the LL genotype of the SERT promoter and carriership of the T allele of the HTR3A 178 C/T polymorphism. This genotype was more prevalent in GERD patients with pathological acid exposure, and in GERD patients with a hypotensive LES we found associations relative to controls as well as patients with a normotensive LES.

In a study by Koutsoumbi et al. ondansetron, a selective 5-HT_3 receptor antagonist, reversed the elevation of LES pressure brought about by erythromycin [38]. When this evidence is reviewed in the light of the associations found in this study, a lower 5-HT availability combined with an attenuated 5-HT3 receptor mediated signaling could alter LES motor function in such a way that the chance of developing GERD increases. HTR3A 178T allele carriers homozygous for the long allele of SERT-P are at increased risk of having hypotensive LES (and therefore GERD). This conclusion is supported by the finding that an association was found with GERD patients with pathological reflux as well. It is possible that the association that was found in the subgroup of GERD patients with pathological acid exposure can be partially explained by the increased risk of a hypotensive LES, however, other conditions such as hiatus hernia and impaired esophageal peristalsis could cause pathological esophageal acid exposure as well.

The GERD patients with physiological reflux but positive symptom association can be viewed as the more viscerally hypersensitive end of the GERD spectrum. It was in subjects with esophageal hypersensitivity that Broekaert et al. found the effect of the SERT-inhibiting drug citalopram [15]. One could expect an association with the SERT promoter polymorphism in this subgroup, however, this group was relatively small and type 2 error could have prevented the discovery of associations.

The TPH2 -703 G/T SNP did not show any association with GERD in this study. The groups of patients included in genetic association studies have to be very large in order to provide sufficient power to entirely rule out weak associations, therefore we cannot conclude that no association with this SNP exists. However, a strong association with GERD of variations in the amount of serotonin synthesized in the central nervous system by TPH2 would have been detected with this study.

This study has some limitations. Controls did neither undergo manometry, 24-hour pH-metry nor upper GI endoscopy. This implies that an unknown number of controls have

asymptomatic manometric abnormalities, pathological acid exposure [39-41]. However, random misclassification is likely to lead to underestimation of effects, possibly preventing the investigator to unveil effects that would have been found when controls would have been phenotyped more extensively. Furthermore the subgroups were rather small. The odds ratios that were found, however, are sufficiently high for interpretation of the outcomes of this study.

In conclusion, we found that the SERT promoter polymorphism combined with the 5-HT_{3A} receptor polymorphism was associated with GERD characterized by pathological acid exposure and a hypotensive LES.

References

1. Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. Am J Gastroenterol 2006; 101(8):1900-1920.

2. Dent J, El Serag HB, Wallander MA, Johansson S. Epidemiology of gastrooesophageal reflux disease: A systematic review. Gut 2005; 54(5):710-717.

3. Holloway RH. The anti-reflux barrier and mechanisms of gastro-oesophageal reflux. Best Pract Res Clin Gastroenterol 2000; 14(5):681-699.

4. Bredenoord AJ, Weusten BLAM, Curvers WL, Timmer R, Smout AJPM. Determinants of perception of heartburn and regurgitation. Gut 2006; 55(3):313-318.

5. Cicala M, Emerenziani S, Caviglia R et al. Intra-oesophageal distribution and perception of acid reflux in patients with non-erosive gastro-oesophageal reflux disease. Aliment Pharmacol Ther 2003; 18(6):605-613.

6. Weusten B, Akkermans L, vanBerge-Henegouwen G, Smout A. Symptom perception in gastroesophageal reflux disease is dependent on spatiotemporal reflux characteristics. Gastroenterology 1995; 108(6):1739-1744.

7. Mehta AJ, De Caestecker JS, Camm AJ, Northfield TC. Sensitization to painful distention and abnormal sensory perception in the esophagus. Gastroenterology 1995; 108(2):311-319.

8. Sarkar S, Aziz Q, Woolf CJ, Hobson AR, Thompson DG. Contribution of central sensitisation to the development of non-cardiac chest pain. Lancet 2000; 356(9236):1154-1159.

9. 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.

10. De Vries DR, Van Herwaarden MA, Baron A, Smout A, Samsom M. Concomitant functional dyspepsia and irritable bowel syndrome decrease health-related quality of life in gastroesophageal reflux disease. Scand J Gastroenterol 2007; 42(8):951-956.

11. Romero Y, Cameron AJ, Locke GR, III et al. Familial aggregation of gastroesophageal reflux in patients with Barrett's esophagus and esophageal adenocarcinoma. Gastroenterology 1997; 113(5):1449-1456.

12. Trudgill NJ, Kapur KC, Riley SA. Familial clustering of reflux symptoms. Am J Gastroenterol 1999; 94(5):1172-1178.

13. Cameron AJ, Lagergren J, Henriksson C, Nyren O, Locke GR, III, Pedersen NL. Gastroesophageal reflux disease in monozygotic and dizygotic twins. Gastroenterology 2002; 122(1):55-59.

14. Mohammed I, Cherkas LF, Riley SA, Spector TT, Trudgill NJ. Genetic influences in gastro-oesophageal reflux disease: a twin study. Gut 2003; 52(8):1085-1089.

15. 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):365-370.

16. Sifrim D, Holloway RH, Tack J et al. Effect of sumatriptan, a 5HT1 agonist, on the frequency of transient lower esophageal sphincter relaxations and gastroesophageal reflux in healthy subjects. Am J Gastroenterol 1999; 94(11):3158-3164.

17. Foster JM, Houghton LA, Whorwell PJ, Morris J. Altered oesophageal motility following the administration of the 5-HT1 agonist, sumatriptan. Aliment Pharmacol Ther 1999; 13(7):927-936.

18. Choung RS, Ferguson DD, Murray JA et al. A novel partial 5HT3 agonist DDP733 after a standard refluxogenic meal reduces reflux events: a randomized, double-blind, placebo-controlled pharmacodynamic study. Aliment Pharmacol Ther 2008; 27(5):404-411.

19. Ruth M, Finizia C, Cange L, Lundell L. The effect of mosapride on oesophageal motor function and acid reflux in patients with gastro-oesophageal reflux disease. Eur J Gastroenterol Hepatol 2003; 15(10):1115-1121.

20. Murphy DL, Lerner A, Rudnick G, Lesch KP. Serotonin Transporter: Gene, Genetic Disorders, and Pharmacogenetics. Mol Interv 2004; 4(2):109-123.

21. Niesler B, Flohr T, Nothen MM et al. Association between the 5' UTR variant C178T of the serotonin receptor gene HTR3A and bipolar affective disorder. Pharmacogenetics 2001; 11(6):471-475.

22. Barrera NP, Herbert P, Henderson RM, Martin IL, Edwardson JM. Atomic force microscopy reveals the stoichiometry and subunit arrangement of 5-HT3 receptors. Proc Natl Acad Sci U S A 2005; 102(35):12595-12600.

23. Liu MT, Rayport S, Jiang Y, Murphy DL, Gershon MD. Expression and function of 5-HT3 receptors in the enteric neurons of mice lacking the serotonin transporter. Am J Physiol Gastrointest Liver Physiol 2002; 283(6):G1398-G1411.

24. Iidaka T, Ozaki N, Matsumoto A et al. A Variant C178T in the Regulatory Region of the Serotonin Receptor Gene HTR3A Modulates Neural Activation in the Human Amygdala. J Neurosci 2005; 25(27):6460-6466.

25. Jonsson EG, Bah J, Melke J et al. Monoamine related functional gene variants and relationships to monoamine metabolite concentrations in CSF of healthy volunteers. BMC Psychiatry 2004; 4:4.

26. Canli T, Congdon E, Gutknecht L, Constable RT, Lesch KP. Amygdala responsiveness is modulated by tryptophan hydroxylase-2 gene variation. J Neural Transm 2005; 112(11):1479-1485.

27. Gutknecht L, Jacob C, Strobel A et al. Tryptophan hydroxylase-2 gene variation influences personality traits and disorders related to emotional dysregulation. Int J Neuropsychopharmacol 2007; 10(3):309-320.

28. Reuter M, Kuepper Y, Hennig J. Association between a polymorphism in the promoter region of the TPH2 gene and the personality trait of harm avoidance. Int J Neuropsychopharmacol 2007; 10(3):401-404.

29. Reuter M, Ott U, Vaitl D, Hennig J. Impaired executive control is associated with a variation in the promoter region of the tryptophan hydroxylase 2 gene. J Cogn Neurosci 2007; 19(3):401-408.

30. de Vries DR, van Herwaarden MA, Smout AJ, Samsom M. Gastroesophageal pressure gradients in gastroesophageal reflux disease: relations with hiatal hernia, body mass index, and esophageal acid exposure. Am J Gastroenterol 2008; 103(6):1349-1354.

31. Wiener GJ, Richter JE, Copper JB, Wu WC, Castell DO. The Symptom Index - A Clinically Important Parameter of Ambulatory 24-Hour Esophageal Ph Monitoring. Am J Gastroenterol 1988; 83(4):358-361.

32. Weusten BL, Roelofs JM, Akkermans LM, Berge-Henegouwen GP, Smout AJ. The symptom-association probability: an improved method for symptom analysis of 24-hour esophageal pH data. Gastroenterology 1994; 107(6):1741-1745.

33. van Lelyveld N, Ter Linde JJM, Schipper M, Samsom M. Candidate genotypes associated with functional dyspepsia. Neurogastroenterol Motil 2008; 20(7):767-773.

34. Mhlanga MM, Malmberg L. Using Molecular Beacons to Detect Single-Nucleotide Polymorphisms with Real-Time PCR. Methods 2001; 25(4):463-471.

35. Zar JH. Biostatistical analysis, 4th edition, Prentice Hall International, Inc, New Jersey. Biostatistical analysis. New Jersey: Prentice Hall International, Inc.; 1999.

36. Lesch KP, Bengel D, Heils A et al. Association of Anxiety-Related Traits with a Polymorphism in the Serotonin Transporter Gene Regulatory Region. Science 1996; 274(5292):1527-1531.

37. Van Den BA, Sleegers K, De ZS et al. Association of brain-specific tryptophan hydroxylase, TPH2, with unipolar and bipolar disorder in a Northern Swedish, isolated population. Arch Gen Psychiatry 2006; 63(10):1103-1110.

38. Koutsoumbi P, Epanomeritakis E, Tsiaoussis J et al. The effect of erythromycin on human esophageal motility is mediated by serotonin receptors. Am J Gastroenterol 2000; 95(12):3388-3392.

39. Ronkainen J, Aro P, Storskrubb T et al. Prevalence of Barrett's esophagus in the general population: an endoscopic study. Gastroenterology 2005; 129(6):1825-1831.

40. Ronkainen J, Aro P, Storskrubb T 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):275-285.

41. Ronkainen J, Talley NJ, Aro P et al. Prevalence of oesophageal eosinophils and eosinophilic oesophagitis in adults: the population-based Kalixanda study. Gut 2007; 56(5):615-620.

Chapter 8.

In GERD patients, mucosal repair associated genes are upregulated in non-inflamed esophageal epithelium

DR de Vries
JJM Ter Linde
MA van Herwaarden
MP Schwartz
P Shephard
MM Geng
AJPM Smout
M Samsom.

Journal of Cellular and Molecular Medicine, *in press*

ABSTRACT

Background: Previous studies addressing the effects of acid reflux and PPI therapy on gene expression in esophageal epithelium concentrated on inflamed tissue. We aimed to determine changes in gene expression in non-inflamed esophageal epithelium of GERD patients.

Methods: We included 20 GERD patients with pathological total 24-h acid exposure of 6-12% and SAP≥95%. Ten patients discontinued PPI treatment (PPI-), ten took pantoprazole 40mg bid (PPI+). Ten age/sex-matched healthy controls were recruited. Biopsies were taken from non-inflamed mucosa 6 cm and 16 cm proximal to the squamocolumnar junction (SCJ). Gene expression profiling of biopsies from 6cm was performed on Human Genome U133 Plus 2.0 arrays (Affymetrix). Genes exhibiting a fold change >1.4 (T-test P-value $<1^{E}$-4) were considered differentially expressed. Results were confirmed by real-time RT-PCR.

Results: In PPI- patients, 92 microarray probesets were deregulated. The majority of the corresponding genes was associated with cell-cell contacts, cytoskeletal reorganization and cellular motility, suggesting facilitation of a migratory phenotype. Genes encoding proteins with anti-apoptotic or anti-proliferative functions or stress-protective functions were also deregulated. No probesets were deregulated in PPI+ patients. QPCR analysis of 20 selected genes confirmed most of the deregulations in PPI- patients, and showed several deregulated genes in PPI+ patients as well. In the biopsies taken at 16cm QPCR revealed no deregulations of the selected genes.

Conclusion: Upon acid exposure, esophageal epithelial cells activate a process globally known as epithelial restitution: up-regulation of anti-apoptotic, anti-oxidant and migration associated genes. Possibly this process helps maintaining barrier function.

INTRODUCTION

Gastroesophageal reflux disease (GERD) is very common in the western world with 10-20% of the population suffering from reflux symptoms at least weekly [1, 2]. Visible mucosal breaks in the esophageal lining, facilitating access of refluxate to submucosal nociceptive nerve endings provide an accepted explanation for symptom generation in GERD patients with esophagitis. However, a substantial number of patients with typical GERD symptoms does not show any macroscopic signs of damage to the esophageal lining and can be classified as non-erosive reflux disease (NERD) patients. A recent report, investigating 999 subjects from the general population, showed that in subjects with daily reflux symptoms, prevalence of esophagitis was only 35.6% [3]. In GERD patients with and without esophagitis, visceral hypersensitivity has been demonstrated in non-inflamed areas [4]. This could be explained by central sensitization of nociceptive pathways [5]. Furthermore, epithelial cells under acid stress may secrete neuroinflammatory substances that can sensitize nociceptive nerve endings peripherally, and cause visceral hypersensitivity in GERD patients with and without esophagitis [6].

Despite the fact that only a minority of GERD patients has esophagitis, studies involving molecular analysis of esophageal epithelium in GERD have, to date, focused mainly on inflamed tissue. In inflamed mucosa, upregulation of cytokines and other pro-inflammatory gene products has been found, e.g. interleukin-8 (IL-8), cyclooxygenase-2 (Cox-2) and nuclear factor kappa B (NF- κB) [7-11]. Interestingly, Isomoto et al. found upregulated expression and increased content of IL-8 and NF-κB in non-inflamed esophageal mucosal biopsies of NERD patients as well [12].

Inflammatory processes, however, are not the initial reaction of esophageal epithelium to acid. Non-inflamed esophageal epithelium possesses a number of defense mechanisms against acid. Inside the epithelial layer, structural and functional defenses provide protection against damage by reflux, e.g. the different junctional complexes between cells (tight junctions (TJs), adhering junctions (AJs) and desmosomes), intercellular glycoconjugates with buffering properties and the epithelial transport proteins that regulate pH and buffering [13]. At a light microscopic level, macroscopically non-inflamed epithelium from GERD patients can display various characteristics, including submucosal papillary elongation, basal layer hyperplasia, infiltration of inflammatory cells, glycogenic acanthosis, hyperemia of the submucosa, thickening of the basement membrane and dilated intercellular spaces [14, 15].

The extent of acid exposure may affect the transcriptional response of the esophageal epithelium. This assumption can be addressed by studying the effect of PPI therapy on

transcription. So far, changes in mRNA expression resulting from PPI therapy have only been determined in esophageal epithelium containing inflitrates of inflammatory cells [11, 12]. Furthermore, insight into the effect of the extent of acid exposure on mRNA expression may be gained by comparing proximal and distal transcription in the esophagus. Weusten et al. showed that acid exposure to the esophageal lining decreases dramatically when a pH-probe is positioned more proximally in the esophagus. This was shown in healthy volunteers and GERD patients [16, 17].

This study aimed, therefore, to investigate the influence of acid reflux on gene expression in non-inflamed esophageal mucosa of GERD patients with pathological esophageal acid exposure, using genome-wide mRNA expression analysis.

METHODS

Patients

From the patients visiting the gastroenterology department at our hospital with recurrent heartburn, acid regurgitation and/or non cardiac chest pain, for at least 2 days per week, lasting 3 months or more, for whom diagnosis of GERD was established by 24-hour esophageal pH recording, twenty consecutive patients characterized by a total esophageal acid exposure time between 6% and 12% were approached.

Patients with severe concomitant diseases, prior esophageal or gastric surgery, esophagitis C or D or Barrett's esophagus, peptic ulcer disease and comorbid conditions that might interfere with esophageal or gastric motility including diabetes mellitus, systemic sclerosis and neurological disorders were non-eligible.

Ten patients discontinued any acid suppressing drugs for the duration of two weeks prior to endoscopy and sampling (PPI-). These patients were permitted to take antacids to alleviate unbearable symptoms with the exception of the 24 hours directly preceding endoscopy. They marked their antacid use on a diary card. The remaining 10 patients were prescribed a fixed PPI dose for two weeks prior to upper GI-endoscopy (PPI+) (pantoprazole 40mg bid) to ensure maximum acid suppression in this group. These patients were randomly assigned to either of the groups, in order of inclusion.

Healthy controls

An advertisement was placed in a local newspaper, and from the people who reacted ten age- and sex-matched healthy controls free of gastrointestinal symptoms or a history of

gastrointestinal disease were included. In conformity with their medical history, none of these subjects had undergone endoscopy before. Should a hiatal hernia or any lesions in the esophagus, stomach or duodenum be found during upper GI endoscopy healthy controls were to be excluded.

Questionnaires

All patients completed a questionnaire assessing reflux symptoms (heartburn, regurgitation, retrosternal pain and belching) in the two weeks prior to endoscopy, modeled after the validated Nepean symptom score [18].

Sample collection

All subjects underwent esophago-gastro-duodenoscopy, all endoscopies were performed by the same gastroenterologist. Six mucosal biopsies were collected at six and sixteen centimeters proximal, respectively, from the squamocolumnar junction (SCJ) (reusable biopsy forceps, 2.2 mm oval cup with spike, Fujinon Medical Holland b.v., Veenendaal, The Netherlands). The biopsy samples were lifted from the forceps with a sterile hypodermic needle. Two biopsies from each location were placed in a sterile 2 ml microcentrifuge tube (Eppendorf, Germany), snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. The remaining two biopsies were fixated in formaldehyde solution for histopathologic evaluation.

RNA extraction

Frozen biopsies were disrupted and homogenized with the Omni μH rotor-stator homogenizer in RLT buffer and subsequently total RNA was extracted using Qiagen RNeasy microkit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Integrity of the samples was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) for distinct 18S and 28S rRNA peaks.

Microarray hybridization

RNA Amplification and Labeling

The RNA extracted from the biopsies acquired at 6cm proximal to the SCJ were used for Affymetrix GeneChip hybridization. Since RNA preparation from biopsy samples did not yield enough total RNA for using the Affymetrix standard labeling protocol, a procedure including an additional PCR amplification step was performed. The samples were amplified as described in the Instruction Manuals from the Microarray Target Amplification Kit and

Microarray Target Purification Kit (Roche Applied Science, Mannheim, Germany): 150 ng of total RNA was used for synthesis of double-stranded cDNA, which was subsequently amplified by PCR. The progress of the PCR reaction was checked by analyzing aliquots of the amplification product from different cycle numbers on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA). 200 ng PCR product from the exponential amplification phase (typically reached after 17 cycles) was then transcribed into biotin-labeled cRNA using the MEGAscript T7 High Yield Transcription Kit (Ambion, Austin, USA), Biotin-11-CTP and Biotin-16-UTP (ENZO Diagnostics, Farmingdale, USA). After incubation for 5 h at 37°C, the transcription product was column-purified with RNeasy Mini Kit (Qiagen, Hilden, Germany). The cRNA was precipitated with ethanol, fragmented at 94°C, pH 8.1, in the presence of Mg2+ and used for Affymetrix chip hybridization.

Affymetrix GeneChip Processing

10 µg of biotin-labeled cRNA was hybridized on Human Genome U133 Plus 2.0 chips (Affymetrix, Santa Clara, USA). Hybridization was performed in a Hybridization Oven 640 (Affymetrix). After 16 h rotation at 45°C, arrays were washed and stained with the Affymetrix Fluidics Station FS-450 using the Fluidics Protocol EukGE-WS2v4. The cRNA was stained with streptavidin-phycoerythrin (SAPE) conjugate (Invitrogen, Carlsbad, USA), incubated with a biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, USA) and then stained again with SAPE. Chip scanning was done with the Laser GeneChip Scanner 3000 7G (Affymetrix).

Quantitative real-time reverse transcriptase polymerase chain reaction (QPCR)

RNA extracted from biopsies taken at both locations was used for QPCR expression analysis. QPCR reactions were performed using TaqMan® low-density arrays (LDAs) (Applied Biosystems, Foster City, CA, USA) using the ABI PRISM® 7900HT Sequence Detection System (AME Bioscience A/S, Norway). From preferably 1 µg of biopsy RNA, or the amount in 10 µl of sample if less was available, cDNA for use on the LDAs was synthesized using the high-capacity cDNA reverse transcription kit according to manufacturer's instructions (Applied Biosystems). 20 µl of cDNA was added with 30 µl of water to 50 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems) and the resulting 100 µl reaction mixtures were loaded onto the LDAs. The LDAs contained 24 different TaqMan® gene expression assays (Applied Biosystems, Foster City, CA, USA) including three housekeeping genes specified below. The 21 remaining assay IDs were Hs00178550_m1 (ABI1), Hs00765651_m1 (ADSS), Hs00177481_m1 (AKAP1), Hs00186689_m1 (ALDH4A1), Hs00761810_s1 (ARF6), Hs00260608_m1 (BRMSL1),

Hs00189232_m1 (COPA), Hs00793391_m1 (CSNK1A1), Hs00158514_m1 (KPNB1), Hs00298519_s1 (MAML3), Hs00172036_m1 (MCL1), Hs00766187_m1 (MCL1), Hs00209335_m1 (MYCBP2), Hs00382970_m1 (PFDN5), Hs00186240_m1 (PPFIBP1), Hs00363974_m1 (PVRL4), Hs00202392_m1 (SLC39A6), Hs00219999_m1 (SPHK2), Hs00370305_m1 (TANK), Hs00197010_m1 (TUBGCP3), Hs00819075_gH (WASF2).

Reference sample

A pooled reference cDNA sample was synthesized using 200 ng of RNA from five healthy controls for use in the normalization calculations, and profiled on the LDAs in the same session of runs as the rest of the samples.

Normalization of PCR data using housekeeping genes

To permit comparison between samples, several housekeeping genes were included in the low-density array to correct for variations in mRNA quality and quantity. These housekeeping genes were chosen after reviewing respective expression values as derived from the microarrays for several well-described housekeeping genes. The housekeeping genes we used were ACTB (beta actin), HMBS (hydroxymethylbilane synthase) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). These genes displayed a stepwise difference in expression level and were not differentially expressed between any of the groups. Housekeeping gene performance was further characterized using the M-value method with the Genorm software package (medgen.ugent.be/genorm) described by Vandesompele et al. [19].

Statistical analysis

Comparison of subject characteristics

Patient characteristics and questionnaire scores were compared between the two groups using Student's T-tests or Chi-Square tests as appropriate, considering a P value of < 0.05 statistically significant.

Computational Analysis of Microarray Data

Affymetrix raw data (CEL files) were analyzed using the Gene Data Expressionist Pro software package in version 2.0 (Gene Data, Basel, Switzerland). (Specifications in the supplementary material.) First, CEL files were subjected to a quality control procedure with the Refiner module which permits global chip quality control with detection and masking of outliers and array defects, and fluorescence gradient correction. Data was condensed with the RMA algorithm [20]. Within the Analyst module signals were first normalized to a logarithmic mean of 1×10^4 in all experiments. An N-way ANOVA was then performed to compare the three groups. No threshold was set for the Affymetrix detection P-value here.

All genes with an ANOVA P-value of $<5\times10^{-6}$ were selected. An Affymetrix detection P-value threshold of < 0.04 was introduced and T-tests for these genes were done for PPI+ versus HC and PPI- patients versus HC (2 T-tests), requiring a minimum of 20% of all experiments per group to deliver valid values with an Affymetrix Detection P-value of < 0.04, otherwise the probeset was excluded. All genes with a fold change of ≥ 1.4 and a T-test P-value of $< 1\times10^{-4}$ were selected.

Analysis of Low Density Array data

TaqMan® LDA results were analyzed with the SDS 2.2.1 software package using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen [21]. The resulting relative quantities (RQs) were compared between groups using one-way ANOVA with Dunnett's post hoc tests. In comparing RQs a P value of < 0.05 was considered statistically significant and a P value of < 0.10 was considered a trend towards significance.

RESULTS

Subjects

The characteristics of the patients and controls are shown in table 1. A total of fourteen healthy controls underwent endoscopy, four healthy controls were excluded upon finding abnormalities (2 grade A esophagitis, 1 grade B esophagitis, 1 Barrett's epithelium).

Table 1. Subject characteristics

	N	male	age	acid exposure(%)*	esophagitis (N)	symptoms (all)†	heartburn, pain‡	belching, regurgitation
HC	10	5	49 (37-63)	N/A	0	N/A	N/A	N/A
PPI -	10	6	46 (25-67)	7.1 (5.7 – 9.9)	7	37 (12-57)	21 (4-32)	16 (0-27)
PPI +	10	4	56 (36-75)	9.3 (6.1 – 11.5)	4	21 (0-44)	10 (0-23)	11 (0-23)

Characteristics of healthy controls (HC), GERD patients off acid suppressive medication (PPI-), and GERD patients using maximum PPI dose (PPI+) two weeks prior to endoscopy. Age, acid exposure and symptoms are displayed as mean (range). Symptom scores cover the two weeks prior to endoscopy and sample collection. N/A: not applicable. *: P = 0.005; †: P = 0.042; ‡: P = 0.017

Age and sex did not differ significantly between the healthy controls and both patient groups and between the patient groups. As expected, PPI- patients had more symptoms in total and

especially more heartburn and retrosternal pain than the PPI+ group during the two weeks prior to biopsy collection. Regurgitation and belching, symptoms that are not treated by acid suppression, were equally prevalent in both patient groups. Esophagitis prevalence did not differ between the PPI- and PPI+ groups. Histologic evaluation confirmed the absence of inflammation in all biopsy locations in all subjects. In three subjects from both patient groups, some infiltrating inflammatory cells were observed, however, in insufficient numbers to qualify as inflammation. Furthermore in some biopsy specimens stromal papillae were close to the mucosal surface, or mild glycogenic acanthosis was present. These findings were equally dispersed between the PPI- and the PPI+ groups. In the healthy controls group one biopsy showed mild glycogenic acanthosis and some lymphocytes.

Differentially expressed genes

Microarray mRNA expression profiling

The results from the microarray mRNA expression profiling are summarized in table 2. The raw data are available as CEL files online at the NCBI Gene Expression Omnibus (GEO) page. Genes differentially expressed relative to healthy controls were found in the biopsies from PPI- patients, but no genes were significantly differentially expressed in the PPI+ patients. Among the differentially expressed genes, groups were discerned with functions that could be tied to cytoskeletal rearrangements and cellular motility or cell-cell contacts.

Table 2. Microarray results, GERD patients off PPI compared to healthy controls

Gene	Description of Gene Function	Affymetrix probe set	Fold change
Genes with functions in cytoskeletal rearrangements and cellular motility			
ARF6	ADP-ribosylation factor 6	214182_at	1.66
ARFGEF1	ADP-ribosylation factor guanine nucleotide-exchange factor 1(brefeldin A-inhibited)	216266_s_at	1.42
MTPN	Myotrophin	223925_s_at	1.55
NCKAP1	NCK-associated protein 1	217465_at	1.62
PLD1	phospholipase D1, phosphatidylcholine-specific	215723_s_at	1.40
PPFIBP1	PTPRF interacting protein, binding protein 1 (liprin beta 1)	203736_s_at	1.45
RPS6KB1 LOC729334 LOC731896	ribosomal protein S6 kinase, 70kDa, polypeptide 1 similar to ribosomal protein S6 kinase, polypeptide 1	211578_s_at	1.46
SLC39A6	solute carrier family 39 (zinc transporter), member 6	1556551_s_at	1.42
TUBGCP3	tubulin, gamma complex associated protein 3	203690_at	1.71
WASF2 WASF4 LOC647909	WAS protein family, member 2 WAS protein family, member 4 similar to WASP-family protein member 4	224563_at	-1.43
ZNF655	zinc finger protein 655	1554726_at	1.49

Genes with functions in cell-cell contacts			
BRMS1L	breast cancer metastasis-suppressor 1-like	224484_s_at	1.72
MPP7	membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)	1564308_a_at	1.60
MYCBP2	MYC binding protein 2	201960_s_at	1.56
PVRL4	poliovirus receptor-related 4	223540_at	1.44
SPHK2	sphingosine kinase 2	40273_at	1.67
DBP	D site of albumin promoter (albumin D-box) binding protein		
Genes with generic functions in epithelial restitution			
ADSS	adenylosuccinate synthase	221761_at	1.55
CSNK1A1	casein kinase 1, alpha 1	208866_at	1.49
MAML3	mastermind-like 3 (Drosophila)	242794_at	1.84
Genes with anti-apoptotic functions			
AKAP1	A kinase (PRKA) anchor protein 1	210625_s_at	1.51
		201674_s_at	1.41
CSNK1A1	casein kinase 1, alpha 1	208866_at	1.49
ETFDH	electron-transferring-flavoprotein dehydrogenase	33494_at	1.49
PPID	peptidylprolyl isomerase D (cyclophilin D)	205530_at	1.44
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	214056_at	1.68
RBMS1	RNA binding motif, single stranded interacting protein 1	207266_x_at	-1.48
		209868_s_at	-1.50
TANK	TRAF family member-associated NFKB activator	210458_s_at	1.65
		207616_s_at	1.46
Genes with anti-proliferative functions			
BRMS1L	breast cancer metastasis-suppressor 1-like	224484_s_at	1.72
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	212515_s_at	1.43
IRF6	interferon regulatory factor6	1552478_a_at	1.49
KPNB1	karyopherin (importin) beta 1	208975_s_at	1.65
		208974_x_at	1.64
		213507_s_at	1.45
RPL29	ribosomal protein L29	213969_x_at	-1.44
		200823_x_at	-1.46
Genes with functions protective against oxidative stress			
ALDH4A1	aldehyde dehydrogenase 4 family, member A1	203722_at	1.51
ALDH7A1	Antiquitin; aldehyde dehydrogenase 7 family, member A1	208951_at	1.49
FAHD1	fumarylacetoacetate hydrolase domain containing 1	227960_s_at	1.44
HAGH	hydroxyacylglutathione hydrolase		
Genes with aspecific or unknown functions			
AGTPBP1	ATP/GTP binding protein 1	204500_s_at	1.47
ASNSD1	asparagine synthetase domain containing 1	217987_at	1.54
ATP5L	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G	207573_x_at	-1.40
		210453_x_at	-1.47
UBE4A	ubiquitination factor E4A (UFD2 homolog, yeast)	208746_x_at	-1.49
ATP8B1	ATPase, Class I, type 8B, member 1	214594_x_at	1.42
ATRX	alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevisiae)	208859_s_at	1.64
LOC728849	similar to transcriptional regulator ATRX isoform 1		
C10orf119	chromosome 10 open reading frame 119	222464_s_at	1.66
C12orf29	chromosome 12 open reading frame 29	213701_at	1.85

C14orf2	chromosome 14 open reading frame 2	210532_s_at	-1.45
CCDC47	coiled-coil domain containing 47	217814_at	1.54
CDV3	CDV3 homolog (mouse)	213548_s_at	1.50
COPA	coatomer protein complex, subunit alpha	208684_at	1.56
CTSB	cathepsin B	227961_at	1.44
DBT	dihydrolipoamide branched chain transacylase E2	205371_s_at	1.59
EIF3J	eukaryotic translation initiation factor 3, subunit J	208985_s_at	1.46
EIF4A2	eukaryotic translation initiation factor 4A, isoform 2	1555996_s_at	1.70
ELF1	E74-like factor 1 (ets domain transcription factor)	233931_at	1.47
FASTKD5 UBOX5	FAST kinase domains 5 U-box domain containing 5	219016_at	1.46
GNG5 CTBS	guanine nucleotide binding protein (G protein), gamma 5 chitobiase, di-N-acetyl-	207157_s_at	-1.43
GNL3L	guanine nucleotide binding protein-like 3 (nucleolar)-like	205010_at	2.30
KIAA0256	KIAA0256 gene product	212451_at	-1.58
LOC400506	similar to TSG118.1	213237_at	1.44
MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)	223849_s_at	1.40
NIPBL	Nipped-B homolog (Drosophila)	207108_s_at	1.46
PDE4DIP LOC727942	phosphodiesterase 4D interacting protein (myomegalin) similar to phosphodiesterase 4D interacting protein isoform 2	214130_s_at	1.59
PITPNA	phosphotidylinositol transfer protein, alpha	201191_at	1.48
PRPF39	PRP39 pre-mRNA processing factor 39 homolog (Yeast)	220553_s_at	1.41
PURB	purine-rich element binding protein B	225120_at	1.52
RETSAT	retinol saturase (all-trans-retinol 13,14-reductase)	1566472_s_at	1.40
RNF10	ring finger protein 10	237062_at	1.68
RNMTL1	RNA methyltransferase like 1	218993_at	1.46
RPE LOC649755	ribulose-5-phosphate-3-epimerase similar to Ribulose-5-phosphate 3-epimerase (HUSSY-17)	216574_s_at	1.40
RPL30 SNORA72	ribosomal protein L30 small nucleolar RNA, H/ACA box 72	200062_s_at	-1.44
RPL37A	ribosomal protein L37a	201429_s_at	-1.41
RPS13 SNORD14A SNORD14B	ribosomal protein S13 small nucleolar RNA, C/D box 14A small nucleolar RNA, C/D box 14B	200018_at	-1.42
RPS18 RP5-1033B10.18	ribosomal protein S18 similar to ribosomal protein S18	201049_s_at	-1.40
RPS24	ribosomal protein S24	200061_s_at	-1.46
RTF1	Rtf1, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)	212302_at	1.56
SAMD8	sterile alpha motif domain containing 8	225950_at	1.87
SAPS3	SAPS domain family, member 3	228105_at	1.62
SART3	squamous cell carcinoma antigen recognised by T cells 3	209127_s_at	1.68
SH3RF2	SH3 domain containing ring finger 2	228892_at	1.47
SLAIN2	SLAIN motif family, member 2	233230_s_at	1.47
SMARCAD1	SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1	223197_s_at	1.45
TCF25	transcription factor 25 (basic helix-loop-helix)	221495_s_at	1.44
TIMM8B	translocase inner mitochondrial membr. 8 homolog B (yeast)	218357_s_at	-1.41

TTF1	transcription termination factor, RNA polymerase I	204772_s_at	1.53
USP24	ubiquitin specific protease 24	212381_at	1.47
ZFYVE16	zinc finger, FYVE domain containing 16	203651_at	1.58
ZKSCAN1	zinc finger with KRAB and SCAN domains 1	1557953_at	1.50
ZNF33A	zinc finger protein 33a (KOX 31)	231864_at	1.45
AK096729 AK092090		1553979_at	1.45
BC015866		1555461_at	1.65

Results are arranged according to function, with the gene names in alphabetical order. For the sake of completeness, genes that have more than one function as represented in this table are mentioned more than once. Grey and white lines belong to different sets of results: either one gene was recognized by multiple probe sets, e.g. AKAP1, or one probe set could have recognized more than one gene, e.g. ATRX/LOC728849. In the case of ATP5L/UBE4A, two genes could both have been recognized by three probesets.

Cytoskeletal rearrangements and cellular motility

The cytoskeleton, consisting of actin, intermediate and tubulin filaments anchored to cell membrane and organelles, is a dynamic structure that enables cellular motion. Several genes with functions in modulating actin or tubulin towards increased cellular motility were found to be differentially expressed. MTPN or V1 regulates the dynamics of actin polymerization by interacting with the capping protein, thereby participating in actin-driven cell movements [22]. PLD1 also regulates cellular migration through the actin cytoskeleton [23]. PPFIBP1 is associated with a migratory phenotype [24]. RPS6KB1 contributes to signaling events of cell migration [25], a process to which SLC39A6 has been tied as well [26]. The remodeling of the actin cytoskeleton is dependent on ARF6 [27]. ARFGEF1 is associated with actin remodeling as well by determining the activation status of ARF6 [28]. Microtubule dynamics are regulated by TUBGCP3 and ZNF655 [29, 30]. PFDN5 or prefoldin is also very important with regard to the integrity of the cytoskeleton as it is essential for formation of functional actin and tubulin [31]. PFDN5 also appeared to be differentially expressed with a fold change of -1.34, just below the threshold. Actin remodeling is in part carried out by a protein complex containing WASF2 and NCKAP1. Abi-1 is essential for the formation and activation of the WASF2/NCKAP1 complex [32, 33]. The fold change of Abi-1 did not exceed the 1.4 threshold of deregulation but showed a fold change of -1.32 nonetheless.

Cell-cell contacts

Migration is accompanied by changes in cell-cell contacts. Intercellular adhesion is mediated by junctional complexes, consisting of the apical tight junctions (TJs), essential for epithelial barrier function, the subapical adherens junctions (AJs) and the basolateral desmosomes. Junctional complexes are linked to cytoskeletal filaments. AJs have a critical role both as

sensors of extracellular stimuli and in regulating the dynamics of epithelial cell layers. Several genes with functions in cell-cell contacts were deregulated. MPP7 affects tight junction function [34]. PVRL4 (nectin-4) is an immunoglobulin-like cell-cell adhesion molecule, which has roles in formation of the E-cadherin-based AJs and subsequent formation of the claudin-based TJs. In addtion to its intercellular adhesion activity, nectin-4 induces activation of Cdc42 and Rac1 small G-proteins, which are well known for their regulation of actin cytoskeleton organization [35, 36]. BRMS1L has a function in migration through regulation of CXCR4 which affects integrins [37, 38], membrane receptors contacting the extracellular matrix, which are important in cell-cell communication and the attachment of cells to their surroundings. Furthermore, SPHK2 catalyses sphingosine-1-phosphate (S1P) formation, an agonist of cell-surface receptors important for cytoskeletal rearrangements en cell migration [39]. MYCBP2 inhibits adenylyl cyclase activity, which may play a role in suppressing cAMP-elevating signals during the time that is needed for relatively slow cellular changes (e.g. cytoskeletal rearrangements) to occur. Of note, MYCBP2 activation/translocation is induced by S1P[40].

Epithelial restitution

Cytoskeletal rearrangements, migration, and regulation of intercellular adhesion can be linked to a process known as epithelial restitution. This is a repair mechanism whereby epithelial continuity is restored and barrier function maintained after superficial injury to the mucosa [41, 42]. During the process of restitution, cells bordering the zone of injury undergo junctional disassembly and flatten. This is viewed as a shift to a migratory phenotype driven by cytoskeletal rearrangements. The flattened migratory epithelial cells spread forward by extending pseudopod-like structures known as lamellipodia, thereby covering the defect in the epithelial layer. Subsequently, cell-cell contacts are reestablished and normal cell shape and phenotype is retained. It has been shown that gap junctional intercellular communication is involved in gastric mucosal restitution following acid-induced injury [43]. Gap junctions are membrane-spanning channels composed of connexins that allow small signaling molecules to pass from cell to cell. Phosphorylation of connexins, mediated by one of the upregulated genes (CSNK1A1) [44], has been implicated in the regulation of gap junctional communication.

The MAML3 gene (mastermind-like 3) codes for a positive regulator of the Notch signaling pathway, which mediates cell-cell communications required for cell fate decisions [45].

Epithelial restitution is a highly energy-dependent process. This might explain the upregulation of adenylosuccinate synthetase (ADSS), an enzyme essential in ATP

production. Accordingly, this enzyme was reported to be upregulated in the healing edges of epithelial wounds [46].

Anti-apoptotic genes

Epithelial restitution is independent of cell proliferation, cell survival however is essential. Several genes with anti-apoptotic functions were upregulated. This may ensure the survival of cells until epithelial restitution is completed.

The upregulated CSNK1A1[47] and PPID[48] have anti-apoptotic effects, while the pro-apoptotic gene RBMS1 was downregulated [49]. AKAP1 targets protein kinase A to mitochondria , consequently PKA-dependent phosphorylation and thereby inactivation of proapoptotic protein BAD is increased and cell survival enhanced [50]. TANK has anti-apoptotic effects by modulating NF-κB activation [51, 52]. MCL1 is a gene of which the N-terminus determines its function: the protein product with the extended N-terminus has strong anti-apoptotic properties and a mild anti-proliferative effect, whereas the protein product with the N-terminal part deleted has a potent anti-proliferative effect while the anti-apoptotic function is less pronounced [53].

Anti-proliferative genes

Several genes with anti-proliferative and/or pro-differentiation properties were found. BRMS1L and DDX3X inhibit cell growth [54, 55], KPNB1 negatively regulates mitotic spindle formation [56, 57] and IRF6 keeps the cell in the G_0 stage of the cell cycle, thus simultaneously promoting differentiation of the cell instead of proliferation [58]. In addition, downregulation of RPL29/HIP is associated with differentiation [59].

Genes with protective roles against oxidative stress

Excessive gastroesophageal reflux is associated with enhanced production of reactive oxygen species (ROS) [60]. Upregulation of ALDH4A1, which has the capacity to reduce ROS generation [61], may protect against reflux-induced oxidative stress. Peroxidation of membrane lipids is one of the mechanisms by which ROS lead to cell damage. Upregulation of ALDH7A1 and HAGH, which function in detoxification including lipid peroxidation products [62, 63], is expected to increase the survival chance of damaged cells.

Two other potentially interesting genes were upregulated with functions independent from epithelial restitution.

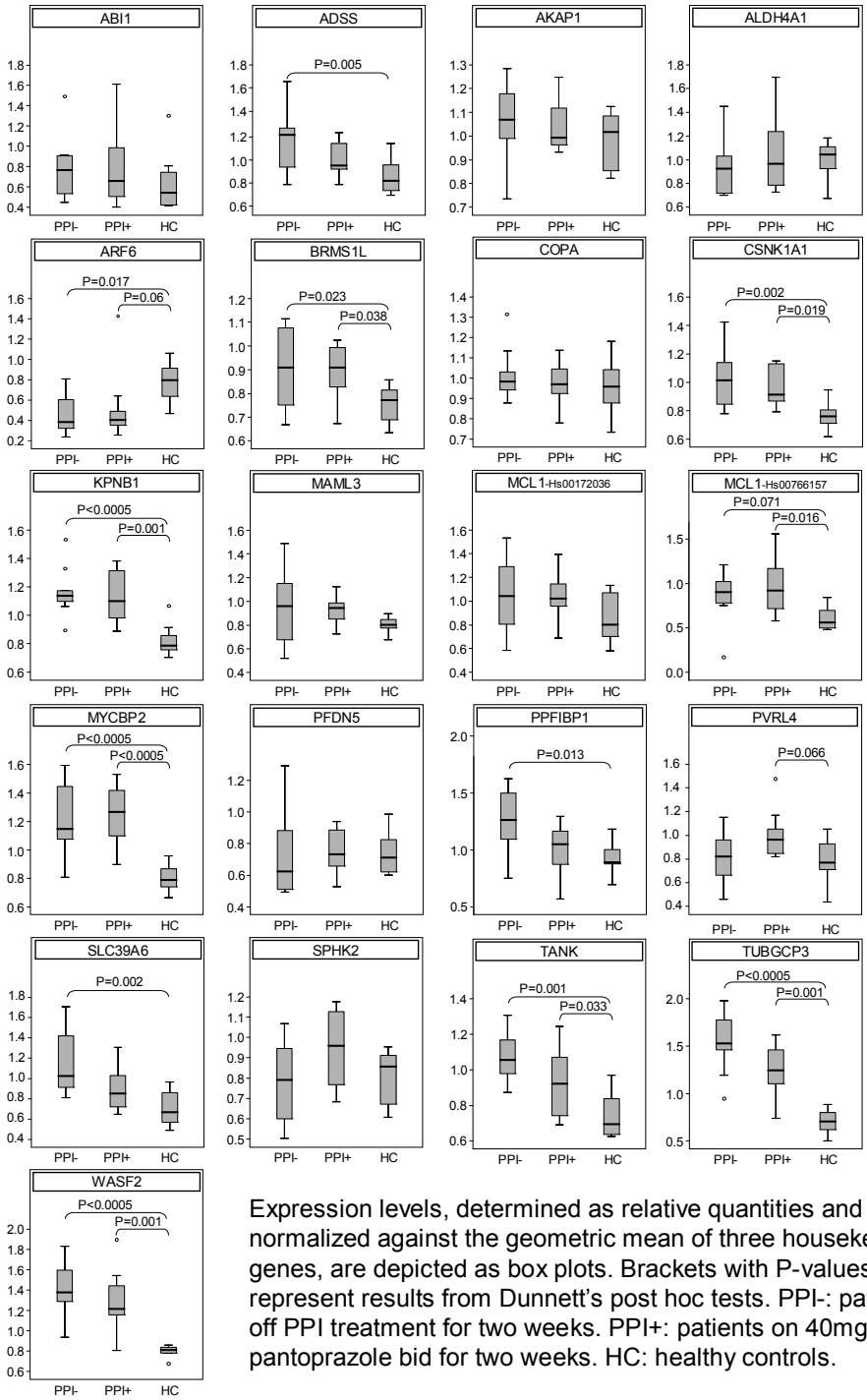
COPA encodes the α-subunit of the coatomer protein complex, involved in intracellular protein transport [64]. The N-terminal amino acids of α-COP are identical to xenin, a neurotensin receptor agonist. Xenin can be cleaved from α-COP by aspartic proteinases

such as pepsin [65]. Interestingly, generation of xenin from its large precursor α-COP increases with acidic pH and exogenous administration of neurotensin reduces LES pressure [66]. Xenin levels have not been quantified in the esophagus to date.
ATP8B1 is a flippase, an enzyme active in restoring membrane symmetry [67], a process which may be involved in maintaining membrane integrity following superficial damage.

TaqMan® Low Density Array validation

Differential expression of 18 genes with putative functions in epithelial restitution was verified using QPCR. The selection of genes covers each of the different aspects of this process described above: cell-cell contacts (CSNK1A1, MAML3, PVRL4), cytoskeletal alterations and cellular motility (Abi-1, ARF6, MYCBP2, PFDN5, PPFIBP1, SLC39A6, SPHK2, TUBGCP3, WASF2), anti-apoptosis (AKAP1, CSNK1A1, MCL1, TANK), anti-proliferation (BRMS1L, KPNB1,), and energy supply (ADSS). In addition, ALDH4A1 and COPA were included in the LDA setup.
Relative quantities of the transcripts are depicted as box plots with post hoc test p-values in figure 1. Eleven genes showed significantly different mRNA expression levels between GERD patients off PPI relative to healthy controls and two genes showed a trend towards significance.
In contrast to the microarray results, in the PPI+ group nine genes were found to be differentially expressed as well, and one gene showed a trend towards significance. Most of the differences, however, were smaller than those in the PPI- group.
The gene MCL1 was represented by two assays on the LDA, because the affymetrix probe set for MCL1, which was detected as significantly different, recognizes two alternative gene products with different functions. Hs00172036_m1 measures the predominantly antiproliferative transcript without the N-terminal end, and Hs00766187_m1 measures the predominantly anti-apoptotic transcript with the N-terminal end intact. Only the latter was differentially expressed in the PPI+ group and showed a trend towards differential expression in the PPI- group.
In addition mRNA from the higher biopsy location, at 16 cm proximal to the SCJ, was subjected to LDA analysis, thereby investigating the expression levels of the selected genes in mucosa that had been exposed to substantially lower amounts of acid [16]. No differential expression of any of the 20 genes was, however, detected at this location in the esophagus.

Figure 1. Expression levels of genes measured with QPCR



Expression levels, determined as relative quantities and normalized against the geometric mean of three housekeeping genes, are depicted as box plots. Brackets with P-values represent results from Dunnett's post hoc tests. PPI-: patients off PPI treatment for two weeks. PPI+: patients on 40mg pantoprazole bid for two weeks. HC: healthy controls.

DISCUSSION

The most important finding of the current study is that in GERD patients on and off PPI treatment, upregulated and downregulated genes were found with functions that inhibit apoptosis, prolong survival, promote differentiation and facilitate the loosening of intercellular contacts, thereby allowing the transformation into a migratory phenotype. This implies, that potential to counter damaging effects of excessive reflux is available in epithelial cells.

The findings of the current study suggest that the first defense of the esophageal epithelium against excess acid reflux consists of only ultrastructural alterations and changes leading to an increased chance of cellular survival. No modifications of the mucosal defense that is already in place (i.e. the mucous layer, the intercellular matrix and the buffer capacity of cells and matrix components) could be deduced from the differentially expressed genes that were found. This does not necessarily mean that humoral defense mechanisms do not play a role in the first response to acid reflux, since the cells that secrete mucus and other buffering substances represent a minority of the epithelial layer. The underrepresentation of cells that secrete matrix components renders differences in mRNA levels in these cells between the investigated groups less likely to be detected.

Despite random assignment, the PPI- group had an average 2.2 percent less acid exposure than the PPI+ group, however, this difference is clinically irrelevant since both means are well above the 6% threshold that defines pathological reflux.

No significantly deregulated genes were found in the comparison of microarray results between healthy controls and PPI+ patients. In contrast, several of the genes selected for QPCR because of differential expression in PPI- patients relative to healthy controls turned out to be significantly deregulated in PPI+ patients as well. These changes in gene expression were, generally, less pronounced than those seen in the PPI- group. This result makes sense, biologically, since acid exposure is not reduced to zero in PPI-treated patients and other caustic components of the refluxate are not targeted by PPIs.

No differences in the transcript levels of the genes selected for validation by QPCR were found in the biopsy specimens from the location that was 10cm more proximal, 16 cm above the SCJ. Since acid exposure can be assumed to be approximately four times less at the proximal relative to the distal site [16], this suggests that the changes in mRNA expression we found are triggered when acid exposure exceeds a certain threshold. The findings of several studies in GERD patients with and without esophagitis (erosive reflux disease (ERD) and non-erosive reflux disease (NERD)) show that reflux episodes that have a larger proximal

extent are more likely to be perceived by the patient [68, 69]. Thus, the mechanisms for perception of these extensive reflux episodes appear to be independent of the gene expression response to acid we describe in the present study. A possible explanation is, that acid perception is primarily a neural response, and since the cell body of the primary afferent neuron is located outside the esophagus, expression differences in these cells would be impossible to detect in the biopsies that were collected in the present study.

The biopsy specimens showed no signs of inflammation on histological evaluation, in patients with and without esophagitis as well as in healthy controls. The explanation for this is that reflux esophagitis is a well-demarcated condition, occurring just proximal to the esophagogastric junction, rarely extending more than a few centimeters in the most severe cases, and these biopsies were taken at 6 cm and 16 cm proximal to the esophagogastric junction.

In the RT-PCR validation results, ARF6 is downregulated and WASF2 is upregulated, opposite to the results of the microarray analysis. The TaqMan® probe for ARF6 has the extension _s1, meaning that this probe recognizes a single-exon transcript, making it possible that part of the signal is generated by genomic DNA contaminating the original RNA sample[70]. The probe for WASF2 has the extension _gH, signifying that this also potentially recognizes genomic DNA[70]. The original samples have been treated with DNase to remove any genomic DNA, minimizing the chance of DNA contamination, however, further validating experiments may be necessary to interpret the findings of the current study.

The epithelial restitution process has been described in esophageal cells in vitro by Jimenez et al. [71, 72], however, despite attempts in several studies, it has not been observed in vivo in esophageal epithelium to date [73]. The results from the present study, however, do indicate a role for epithelial restitution in the first response to excessive acid exposure of the esophageal epithelium. The finding that so many genes that have anti-proliferative and anti-apoptotic functions were deregulated, in combination with the deregulation of genes that facilitate transition into a migratory phenotype are concordant with the two key events of the epithelial restitution process: cell survival and subsequent migration, in reaction to minor breaches in the epithelial continuity. The epithelial restitution process has been well-characterized in intestinal columnar epithelium, however no events resembling epithelial restitution have been described in esophageal epithelium to date. A possible explanation is that the esophagus is lined with pseudostratified squamous epithelium, in which minor migratory movements are hard to discern, especially when no such concept has been postulated yet. Further studies in vitro and vivo could clarify the phenotypic correlate of the gene expression changes we found.

No genes encoding cytokines, other pro-inflammatory substances or upregulators thereof were found to be differentially expressed in our patients. On the contrary, upregulation of the TANK gene which encodes a protein that inhibits NF-κB activation may exert an anti-inflammatory effect. At the AGA Digestive Disease Week of 2005, Yoshida et al. presented results of a microarray study conducted in NERD patients. They did report up-regulation of a wide variety of pro-inflammatory substances in the epithelial cells they had selectively studied with the help of laser capture microdissection [74]. However, this group did not confirm results with RT-PCR, and furthermore the biopsies studied by Yoshida et al were taken closer to the squamocolumnar junction. Inflammatory infiltrates are present in a large portion of NERD patients' esophagus near the SCJ [75], and these infiltrating cells could have exerted an influence on gene expression in the epithelial cells. In another study using microarray expression profiling with the same arrays as were used in the present study, Ostrowski et al compared biopsy specimens from non-inflamed esophageal epithelium of NERD patients, patients with esophagitis and patients with Barrett's esophagus. They found a distinct genetic signature for each of these patient groups. Unfortunately no GERD-free controls were included in that study, which makes comparing their work to the current study difficult [76]. Furthermore, no 24-hour pH-monitoring was included in the diagnostic work-up of the included subjects, possibly introducing phenotypical heterogeneity.

No upregulation of neuroinflammatory substances was detected. It can be hypothesized that protons, that are able to reach the nerve endings in the intra- and subcellular layer in larger amounts due to increased paracellular permeability, are the main mechanism for the heightened visceral sensitivity that has been found in GERD patients.

The COPA gene could not be confirmed by QPCR, however, this does not rule out xenin expression in the esophagus, and a study design incorporating immunohistochemistry is needed to clarify this. It would also be interesting to further investigate the ATP8B1 gene, since no expression in the esophagus of this important gene has been described thus far. The CEL files that have been made available online provide an excellent resource for researchers to verify the esophageal expression of genes of their interest, and could instigate numerous new studies.

One could argue that the groups in the present study were relatively small, and that the results should be interpreted with caution. Two important preventive measures were taken, however, to maximize reliability: firstly the detection thresholds for the microarray results were set to very strict levels, in order to ascertain that only significant expression differences would be detected. Secondly, RT-PCR analysis confirmed the majority of the found differences, providing a second measure of validity of the results. The current design was

chosen to maximize results within the ethical and financial boundaries we faced, and was meant as a pilot work for future investigations.

Further studies are needed to investigate the epithelial restitution process in the esophagus to clarify, for instance, the epithelial response in different groups of GERD patients, e.g. NERD patients or patients with severe esophagitis or Barrett's esophagus, and to investigate whether these genetic changes can be tied to a morphologic correlate.

In conclusion, this study is the first to provide validated genome-wide expression data about non-inflamed esophageal epithelium in GERD patients compared to healthy controls. The results point towards activation by acid reflux of the process we know as epithelial restitution, which has not been described before in the esophagus.

References

1. Camilleri M, Dubois D, Coulie B et al. Prevalence and socioeconomic impact of upper gastrointestinal disorders in the United States: results of the US Upper Gastrointestinal Study. Clin Gastroenterol Hepatol 2005; 3(6):543-552.

2. Dent J, El Serag HB, Wallander MA, Johansson S. Epidemiology of gastrooesophageal reflux disease: A systematic review. Gut 2005; 54(5):710-717.

3. Ronkainen J, Aro P, Storskrubb T et al. Gastro-oesophageal reflux symptoms and health-related quality of life in the adult general population - the Kalixanda study. Aliment Pharmacol Ther 2006; 23(12):1725-1733.

4. 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.

5. Fass R, Naliboff B, Higa L et al. Differential effect of long-term esophageal acid exposure on mechanosensitivity and chemosensitivity in humans. Gastroenterology 1998; 115(6):1363-1373.

6. Holzer P. Sensory neurone responses to mucosal noxae in the upper gut: relevance to mucosal integrity and gastrointestinal pain. Neurogastroent Motil 2002; 14(5):459-475.

7. Fitzgerald RC, Onwuegbusi BA, Bajaj-Elliott M, Saeed IT, Burnham WR, Farthing MJG. Diversity in the oesophageal phenotypic response to gastro-oesophageal reflux: immunological determinants. Gut 2002; 50(4):451-459.

8. Hamoui N, Peters JH, Schneider S et al. Increased acid exposure in patients with gastroesophageal reflux disease influences cyclooxygenase-2 gene expression in the squamous epithelium of the lower esophagus. Arch Surg 2004; 139(7):712-717.

9. Matthews P, Aziz Q, Facer P, Davis J, Thompson D, Anand P. Increased expression of the capsaicin receptor TRPV1 in the inflamed human esophagus. Gastroenterology 2004; 126(4):A427.

10. Souza RF, Shewmake KL, Shen Y et al. Differences in ERK Activation in Squamous Mucosa in Patients Who Have Gastroesophageal Reflux Disease with and without Barrett's Esophagus. Am J Gastroenterol 2005; 100(3):551-559.

11. Yoshida N, Uchiyama K, Kuroda M et al. Interleukin-8 expression in the esophageal mucosa of patients with gastroesophageal reflux disease. Scand J Gastroenterol 2004; 39(9):816-822.

12. Isomoto H, Saenko VA, Kanazawa Y et al. Enhanced expression of interleukin-8 and activation of nuclear factor kappa-B in endoscopy-negative gastroesophageal reflux disease. Am J Gastroenterol 2004; 99(4):589-597.

13. Orlando RC. oesophageal mucosal resistance. Aliment Pharmacol Ther 1998; 12(3):191-197.

14. Takubo K, Honma N, Aryal G et al. Is there a set of histologic changes that are invariably reflux associated? Arch Pathol Lab Med 2005; 129(2):159-163.

15. Vieth M, Fiocca R, Haringsma J et al. Radial distribution of dilated intercellular spaces of the esophageal squamous epithelium in patients with reflux disease exhibiting discrete endoscopic lesions. Digest Dis 2004; 22(2):208-212.

16. Weusten B, Akkermans L, vanBerge-Henegouwen G, Smout A. Symptom perception in gastroesophageal reflux disease is dependent on spatiotemporal reflux characteristics. Gastroenterology 1995; 108(6):1739-1744.

17. Weusten BL, Akkermans LM, vanBerge-Henegouwen GP, Smout AJ. Spatiotemporal characteristics of physiological gastroesophageal reflux. Am J Physiol Gastrointest Liver Physiol 1994; 266(3):G357-G362.

18. Talley NJ, Verlinden M, Jones M. Validity of a new quality of life scale for functional dyspepsia: a United States multicenter trial of the Nepean Dyspepsia Index. American Journal of Gastroenterology 1999; 94(9):2390-2397.

19. Vandesompele J, De PK, Pattyn F et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3(7):RESEARCH0034.

20. Irizarry RA, Hobbs B, Collin F et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostat 2003; 4(2):249-264.

21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method
1. Methods 2001; 25(4):402-408.

22. Taoka M, Ichimura T, Wakamiya-Tsuruta A et al. V-1, a protein expressed transiently during murine cerebellar development, regulates actin polymerization via interaction with capping protein. J Biol Chem 2003; 278(8):5864-5870.

23. Kim JH, Kim HW, Jeon H, Suh PG, Ryu SH. Phospholipase D1 regulates cell migration in a lipase activity-independent manner. J Biol Chem 2006; 281(23):15747-15756.

24. Kriajevska M, Fischer-Larsen M, Moertz E et al. Liprin beta 1, a member of the family of LAR transmembrane tyrosine phosphatase-interacting proteins, is a new target for the metastasis-associated protein S100A4 (Mts1). J Biol Chem 2002; 277(7):5229-5235.

25. Berven LA, Willard FS, Crouch MF. Role of the p70(S6K) pathway in regulating the actin cytoskeleton and cell migration. Exp Cell Res 2004; 296(2):183-195.

26. Zhao L, Chen W, Taylor KM, Cai B, Li X. LIV-1 suppression inhibits HeLa cell invasion by targeting ERK1/2-Snail/Slug pathway. Biochem Biophys Res Commun 2007; 363(1):82-88.

27. Klein S, Franco M, Chardin P, Luton F. Role of the Arf6 GDP/GTP cycle and Arf6 GTPase-activating proteins in actin remodeling and intracellular transport. J Biol Chem 2006; 281(18):12352-12361.

28. Jones HD, Moss J, Vaughan M. BIG1 and BIG2, brefeldin A-inhibited guanine nucleotide-exchange factors for ADP-ribosylation factors. Methods Enzymol 2005; 404:174-184.

29. Raynaud-Messina B, Merdes A. Gamma-tubulin complexes and microtubule organization. Curr Opin Cell Biol 2007; 19(1):24-30.

30. Allingham JS, Sproul LR, Rayment I, Gilbert SP. Vik1 modulates microtubule-Kar3 interactions through a motor domain that lacks an active site. Cell 2007; 128(6):1161-1172.

31. Simons CT, Staes A, Rommelaere H, Ampe C, Lewis SA, Cowan NJ. Selective contribution of eukaryotic prefoldin subunits to actin and tubulin binding. J Biol Chem 2004; 279(6):4196-4203.

32. Takenawa T, Miki H. WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. Journal of Cell Science 2001; 114(10):1801-1809.

33. Innocenti M, Zucconi A, Disanza A et al. Abi1 is essential for the formation and activation of a WAVE2 signalling complex. Nat Cell Biol 2004; 6(4):319-327.

34. Stucke VM, Timmerman E, Vandekerckhove J, Gevaert K, Hall A. The MAGUK protein MPP7 binds to the polarity protein hDlg1 and facilitates epithelial tight junction formation. Mol Biol Cell 2007; 18(5):1744-1755.

35. Tachibana K, Nakanishi H, Mandai K et al. Two cell adhesion molecules, nectin and cadherin, interact through their cytoplasmic domain-associated proteins. J Cell Biol 2000; 150(5):1161-1176.

36. Tanaka Y, Nakanishi H, Kakunaga S et al. Role of nectin in formation of E-cadherin-based adherens junctions in keratinocytes: analysis with the N-cadherin dominant negative mutant. Mol Biol Cell 2003; 14(4):1597-1609.

37. Huang YC, Hsiao YC, Chen YJ, Wei YY, Lai TH, Tang CH. Stromal cell-derived factor-1 enhances motility and integrin up-regulation through CXCR4, ERK and NF-kappaB-dependent pathway in human lung cancer cells. Biochem Pharmacol 2007; 74(12):1702-1712.

38. Yang J, Zhang B, Lin Y, Yang Y, Liu X, Lu F. Breast cancer metastasis suppressor 1 inhibits SDF-1alpha-induced migration of non-small cell lung cancer by decreasing CXCR4 expression. Cancer Lett 2008.

39. Hait NC, Sarkar S, Le SH et al. Role of sphingosine kinase 2 in cell migration toward epidermal growth factor. J Biol Chem 2005; 280(33):29462-29469.

40. Pierre SC, Hausler J, Birod K, Geisslinger G, Scholich K. PAM mediates sustained inhibition of cAMP signaling by sphingosine-1-phosphate. EMBO J 2004; 23(15):3031-3040.

41. Mammen JM, Matthews JB. Mucosal repair in the gastrointestinal tract. Crit Care Med 2003; 31(8 Suppl):S532-S537.

42. Lacy ER, Ito S. Rapid epithelial restitution of the rat gastric mucosa after ethanol injury. Lab Invest 1984; 51(5):573-583.

43. Takahashi N, Joh T, Yokoyama Y et al. Importance of gap junction in gastric mucosal restitution from acid-induced injury. J Lab Clin Med 2000; 136(2):93-99.

44. Solan JL, Lampe PD. Connexin phosphorylation as a regulatory event linked to gap junction channel assembly. Biochim Biophys Acta 2005; 1711(2):154-163.

45. Lin SE, Oyama T, Nagase T, Harigaya K, Kitagawa M. Identification of new human mastermind proteins defines a family that consists of positive regulators for notch signaling. J Biol Chem 2002; 277(52):50612-50620.

46. Gassmann MG, Stanzel A, Werner S. Growth factor-regulated expression of enzymes involved in nucleotide biosynthesis: a novel mechanism of growth factor action. Oncogene 1999; 18(48):6667-6676.

47. Izeradjene K, Douglas L, Delaney AB, Houghton JA. Casein kinase I attenuates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by regulating the recruitment of fas-associated death domain and procaspase-8 to the death-inducing signaling complex. Cancer Res 2004; 64(21):8036-8044.

48. Machida K, Ohta Y, Osada H. Suppression of apoptosis by cyclophilin D via stabilization of hexokinase II mitochondrial binding in cancer cells. J Biol Chem 2006; 281(20):14314-14320.

49. Nomura J, Matsumoto K, Iguchi-Ariga SM, Ariga H. Mitochondria-independent induction of Fas-mediated apoptosis by MSSP. Oncol Rep 2005; 14(5):1305-1309.

50. Affaitati A, Cardone L, De CT et al. Essential role of A-kinase anchor protein 121 for cAMP signaling to mitochondria. J Biol Chem 2003; 278(6):4286-4294.

51. Pomerantz JL, Baltimore D. NF-kappaB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. EMBO J 1999; 18(23):6694-6704.

52. Rothe M, Xiong J, Shu HB, Williamson K, Goddard A, Goeddel DV. I-TRAF is a novel TRAF-interacting protein that regulates TRAF-mediated signal transduction. Proc Natl Acad Sci U S A 1996; 93(16):8241-8246.

53. Germain M, Duronio V. The N terminus of the anti-apoptotic BCL-2 homologue MCL-1 regulates its localization and function. J Biol Chem 2007; 282(44):32233-32242.

54. Chao CH, Chen CM, Cheng PL, Shih JW, Tsou AP, Lee YH. DDX3, a DEAD box RNA helicase with tumor growth-suppressive property and transcriptional regulation activity of the p21waf1/cip1 promoter, is a candidate tumor suppressor. Cancer Res 2006; 66(13):6579-6588.

55. Nikolaev AY, Papanikolaou NA, Li M, Qin J, Gu W. Identification of a novel BRMS1-homologue protein p40 as a component of the mSin3A/p33(ING1b)/HDAC1 deacetylase complex. Biochem Biophys Res Commun 2004; 323(4):1216-1222.

56. Harel A, Forbes DJ. Importin beta: Conducting a much larger cellular symphony. Molecular Cell 2004; 16(3):319-330.

57. Tahara K, Takagi M, Ohsugi M et al. Importin-beta and the small guanosine triphosphatase Ran mediate chromosome loading of the human chromokinesin Kid. J Cell Biol 2008; 180(3):493-506.

58. Bailey CM, Abbott DE, Margaryan NV, Khalkhali-Ellis Z, Hendrix MJ. IRF6 Promotes Cell Cycle Arrest and is Regulated by the Proteasome in a Cell Cycle-dependent Manner. Mol Cell Biol 2008.

59. Miller SA, Brown AJ, Farach-Carson MC, Kirn-Safran CB. HIP/RPL29 down-regulation accompanies terminal chondrocyte differentiation. Differentiation 2003; 71(6):322-336.

60. Wetscher GJ, Hinder RA, Bagchi D et al. Reflux esophagitis in humans is mediated by oxygen-derived free radicals. Am J Surg 1995; 170(6):552-556.

61. Yoon KA, Nakamura Y, Arakawa H. Identification of ALDH4 as a p53-inducible gene and its protective role in cellular stresses. J Hum Genet 2004; 49(3):134-140.

62. Vasiliou V, Pappa A, Estey T. Role of human aldehyde dehydrogenases in endobiotic and xenobiotic metabolism. Drug Metab Rev 2004; 36(2):279-299.

63. Thornalley PJ. Glyoxalase I--structure, function and a critical role in the enzymatic defence against glycation. Biochem Soc Trans 2003; 31(Pt 6):1343-1348.

64. Schroder-Kohne S, Letourneur F, Riezman H. Alpha-COP can discriminate between distinct, functional di-lysine signals in vitro and regulates access into retrograde transport. J Cell Sci 1998; 111 (Pt 23):3459-3470.

65. Feurle GE. Xenin--a review. Peptides 1998; 19(3):609-615.

66. Theodorsson-Norheim E, Thor K, Rosell S. Relation between lower esophageal sphincter (LES) pressure and the plasma concentration of neurotensin during intravenous infusion of neurotensin(1-13) and (Gln4)neurotensin(1-13) in man. Acta Physiol Scand Suppl 1983; 515:29-35.

67. Paulusma CC, Folmer DE, Ho-Mok KS et al. ATP8B1 requires an accessory protein for endoplasmic reticulum exit and plasma membrane lipid flippase activity. Hepatology 2008; 47(1):268-278.

68. Bredenoord AJ, Weusten BLAM, Curvers WL, Timmer R, Smout AJPM. Determinants of perception of heartburn and regurgitation. Gut 2006; 55(3):313-318.

69. Schey R, Shapiro M, Navarro-Rodriguez T et al. Comparison of the Different Characteristics of Sensed Reflux Events Among Different Heartburn Groups. J Clin Gastroenterol 2008.

70. Lazaruk K, Wang Y, Zhong J et al. The Design Process for a New Generation of Quantitative Gene Expression Analysis Tools - TaqMan® Probe-based Assays for Human and Other Model Organism Genes. Applied Biosystems . 2006.
Ref Type: Internet Communication

71. Jimenez P, Lanas A, Piazuelo E, Esteva F. Effect of growth factors and prostaglandin E2 on restitution and proliferation of rabbit esophageal epithelial cells. Dig Dis Sci 1998; 43(10):2309-2316.

72. Jimenez P, Lanas A, Piazuelo E, Esteva F. Effects of extracellular pH on restitution and proliferation of rabbit oesophageal epithelial cells. Aliment Pharmacol Ther 1999; 13(4):545-552.

73. Tobey NA, Powell DW, Schreiner VJ, Orlando RC. Serosal bicarbonate protects against acid injury to rabbit esophagus. Gastroenterology 1989; 96(6):1466-1477.

74. Yoshida N, Kuroda M, Uchiyama K et al. Microarray analysis of transcriptome in patients with non-erosive reflux disease. Gastroenterology 128[4 s2]. 2005.
Ref Type: Abstract

75. Zentilin P, Savarino V, Mastracci L et al. Reassessment of the diagnostic value of histology in patients with GERD, using multiple biopsy sites and an appropriate control group. Am J Gastroenterol 2005; 100(10):2299-2306.

76. Ostrowski J, Rubel T, Wyrwicz LS et al. Three clinical variants of gastroesophageal reflux disease form two distinct gene expression signatures. J Mol Med 2006; 84(10):872-882.

Chapter 9.

Differential gene expression in duodenum of GERD patients: upregulations in lipid metabolism and chylomicron production and transport point towards enhanced ApoA-IV and CCK signalling

DR de Vries
JJM Ter Linde
MA van Herwaarden
P Shephard
MM Geng
MP Schwartz
AJPM Smout
M Samsom.

Manuscript in preparation

ABSTRACT

Background: Several studies have shown that the duodenum plays a role in the pathophysiology of GERD symptoms. Signaling from the duodenum affects TLESR frequency, LES pressure and esophageal perception. Abnormalities in duodenal gene expression underlying altered sensorimotor function of the esophagus have not been reported to date. We therefre aimed to identify differentially expressed genes in duodenal mucosal biopsies from GERD patients compared to healthy volunteers (HV).

Methods: Twenty symptomatic GERD patients (10 M/F, mean age 52) with a total 24-h acid exposure of 6-12% and a SAP equal to or greater than 95% were selected from recent pH-metry referrals. Patients with a history of organic disease and/or surgery affecting upper GI tract sensitivity and/or motility were excluded. Ten patients discontinued PPI treatment and ten patients took maximum dose PPI, both two weeks prior to sampling. Ten age- and sex-matched HV without gastrointestinal symptoms or history of symptoms were recruited from the general population. Mucosal biopsies were taken in the pars descendens duodeni and snap-frozen. RNA was extracted and profiled on Affymetrix Human Genome U133 Plus 2.0 array. Genes exhibiting a fold change >1.4 (t-test p-value <1E-4) were considered differentially expressed. A subset of 21 differentially expressed genes was selected for confirmatory RT-PCR using TaqMan low density arrays, and compared to three housekeeping genes.

Results: Whole genome expression profiling revealed that 30 and 23 genes were higher respectively lower expressed in GERD patients off PPIs relative to HVs. In GERD patients on PPIs 42 upregulated and 5 downregulated genes were found, taking HVs as a reference. The majority of these genes were associated with lipid absorption, triglyceride resynthesis and intracellular vesicular transport, which are rate-limiting processes for chylomicron production and secretion. Differential expression of eleven upregulated genes was confirmed by RT-PCR.

Conclusion: Gene expression in the duodenum of GERD patients is different from gene expression in healthy controls. Our results suggest that in GERD patients chylomicron production and secretion in the duodenal mucosa is enhanced.

INTRODUCTION

Gastroesophageal reflux disease (GERD) is defined as a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications [1]. The classical symptoms are heartburn, regurgitation and non-cardiac retrosternal pain. GERD is very common in the western world with 10-20% of the population suffering from reflux symptoms at least weekly [2, 3]. The pathophysiology of GERD is only partially understood.

Feedback from the duodenum after lipid infusion causes alterations in pressure and transient relaxations of the lower esophageal sphincter (TLESRs). Holloway et al showed that intraduodenal fat infusions increased the proportion of total TLESRs that was accompanied by acid reflux [4]. Ledeboer et al showed that LES pressure was lowered by both long chain and medium chain fatty acids, but that only the long chain fatty acid response was mediated by cholecystokinin (CCK) [5]. Furthermore, feedback from the duodenum regulating sensory processes in the esophagus plays a role in GERD. Meyer et al showed that infusion of lipids in the duodenum intensifies the sensations caused by acid in the esophagus; the time needed to evoke heartburn by intraesophageal infusions of hydrochloric acid infusions of increasing pH was shorter during simultaneous lipid perfusion of the duodenum, and the intensity and severity of the symptoms were greater as well [6]. This feedback connection was observed in patients with functional dyspepsia as well, a syndrome with a substantial overlap with GERD [7]: Barrera et al showed that duodenal fat infusion intensified the symptoms these patients experienced upon gastric distension [8].

Heightened visceral sensitivity plays a role in GERD symptom generation [9], and previous studies have shown that visceral sensitivity often is altered on more than one location in the gastrointestinal tract [10].

This study aimed to compare gene expression in the duodenal mucosa of patients with moderately severe proven GERD on and off PPI to gene expression in the duodenal mucosa of healthy controls.

METHODS

Subjects

Patients

Twenty patients were selected from individuals visiting the gastroenterology department at our hospital with recurrent heartburn, acid regurgitation and/or non cardiac chest pain, for at least 2 days per week, lasting 3 months or more, for whom diagnosis of GERD was established by 24-hour esophageal pH recording. Patients with a total esophageal acid exposure time between 6% and 12% were selected.

Patients with severe concomitant diseases, prior esophageal or gastric surgery, esophagitis C or D or Barrett's esophagus, peptic ulcer disease and comorbid conditions that might interfere with esophageal or gastric motility including diabetes mellitus, systemic sclerosis and neurological disorders were non-eligible.

Ten randomly selected patients discontinued any acid suppressing drugs for the duration of two weeks prior to endoscopy and sampling (PPI-). These patients were permitted to take antacids to alleviate unbearable symptoms with the exception of the 24 hours directly preceding endoscopy. They marked their antacid use on a diary card. The remaining 10 patients were prescribed a fixed PPI dose for two weeks prior to upper GI-endoscopy (PPI+) (pantoprazole 40mg bid) to ensure maximum acid suppression in this group.

Healthy controls

Ten age- and sex-matched healthy controls free of gastrointestinal symptoms or a history of gastrointestinal disease were included. Should a hiatal hernia or any lesions in the esophagus, stomach or duodenum be found during upper GI endoscopy healthy controls were to be excluded**.**

Questionnaires

All patients completed a questionnaire assessing reflux symptoms (heartburn, regurgitation, retrosternal pain and belching) in the two weeks prior to endoscopy, modeled after the validated Nepean symptom score [11].

Sample collection

All subjects underwent esophago-gastro-duodenoscopy, all endoscopies were performed by the same gastroenterologist. Three mucosal biopsies were collected in the pars descendens of the duodenum (reusable biopsy forceps, 2.2 mm oval cup with spike, Fujinon Medical Holland b.v., Veenendaal, The Netherlands). The biopsy samples were lifted from the

forceps with a sterile hypodermic needle. The biopsies were placed in a sterile 2 ml microcentrifuge tube (Eppendorf, Germany), snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction

Frozen biopsies were disrupted and homogenized with the Omni µH rotor-stator homogenizer in RLT buffer and subsequently total RNA was extracted using Qiagen RNeasy microkit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Integrity of the samples was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) for distinct 18S and 28S rRNA peaks.

Microarray hybridization

RNA Amplification and Labeling

The RNA extracted from the biopsies was used for Affymetrix GeneChip hybridization, specified below. Since RNA preparation from biopsy samples did not yield enough total RNA for using the Affymetrix standard labeling protocol, a procedure including an additional PCR amplification step was performed. The samples were amplified as described in the Instruction Manuals from the Microarray Target Amplification Kit and Microarray Target Purification Kit (Roche Applied Science, Mannheim, Germany): 150 ng of total RNA was used for synthesis of double-stranded cDNA, which was subsequently amplified by PCR. The progress of the PCR reaction was checked by analyzing aliquots of the amplification product from different cycle numbers on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA). 200 ng PCR product from the exponential amplification phase (typically reached after 17 cycles) was then transcribed into biotin-labeled cRNA using the MEGAscript T7 High Yield Transcription Kit (Ambion, Austin, USA), Biotin-11-CTP and Biotin-16-UTP (ENZO Diagnostics, Farmingdale, USA). After incubation for 5 h at 37°C, the transcription product was column-purified with RNeasy Mini Kit (Qiagen, Hilden, Germany). The cRNA was precipitated with ethanol, fragmented at 94°C, pH 8.1, in the presence of Mg^{++} and used for Affymetrix chip hybridization.

Affymetrix GeneChip Processing

10 µg of biotin-labeled cRNA was hybridized on Human Genome U133 Plus 2.0 chips (Affymetrix, Santa Clara, USA). Hybridization was performed in a Hybridization Oven 640 (Affymetrix). After 16 h rotation at 45°C, arrays were washed and stained with the Affymetrix Fluidics Station FS-450 using the Fluidics Protocol EukGE-WS2v4. The cRNA was stained

with streptavidin-phycoerythrin (SAPE) conjugate (Invitrogen, Carlsbad, USA), incubated with a biotinylated anti-streptavidin antibody
(Vector Laboratories, Burlingame, USA) and then stained again with SAPE. Chip scanning was done with the Laser GeneChip Scanner 3000 7G (Affymetrix).

Quantitative real-time reverse transcriptase polymerase chain reaction (QPCR)

RNA extracted from the biopsies was used for QPCR expression analysis. QPCR reactions were performed using TaqMan® low-density arrays (LDAs) (Applied Biosystems, Foster City, CA, USA) using the ABI PRISM® 7900HT Sequence Detection System (AME Bioscience A/S, Norway). From preferably 1 µg of biopsy RNA, or the amount in 10 µl of sample if less was available, cDNA for use on the LDAs was synthesized using the high-capacity cDNA reverse transcription kit according to manufacturer's instructions (Applied Biosystems). 20 µl of cDNA was added with 30 µl of water to 50 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems) and the resulting 100 µl reaction mixtures were loaded onto the LDAs. The LDAs contained 24 different TaqMan® gene expression assays (Applied Biosystems, Foster City, CA, USA) including three housekeeping genes specified below. The 21 remaining assay IDs were Hs00197410_m1 (AGPAT2), Hs00163626_m1 (ALDOB), Hs00357578_m1 (ALPI), Hs00384501_m1 (ASAHL), Hs00376009_m1 (COMTD1), Hs00240921_m1 (CYB5R3), Hs00166864_m1 (HEXB), Hs00381934_m1 (HIBADH), Hs00180741_m1 (JTB), Hs00210880_m1 (LSR), Hs00827792_m1 (MPZL1), Hs00748915_s1 (PFN1), Hs00359986_m1 (PGLS), Hs00198499_m1 (PGRMC1), Hs00170356_m1 (PPAP2A), Hs00162467_m1 (SURF1), Hs00193899_m1 (MED16), Hs00292741_m1 (TMEM55B), Hs00427749_m1 (VAPA), Hs00270351_m1 (VIPR1), Hs00559914_m1 (YKT6).

Reference sample

A pooled reference cDNA sample was synthesized using 200 ng of RNA from five healthy controls for use in the normalization calculations, and profiled on the LDAs in the same session of runs as the rest of the samples.

Normalization of PCR data using housekeeping genes

To permit comparison between samples, several housekeeping genes were included in the low-density array to correct for variations in mRNA quality and quantity. These housekeeping genes were chosen after reviewing respective expression values as derived from the microarrays for several well-described housekeeping genes. The housekeeping genes we used were ACTB (beta actin), HMBS (hydroxymethylbilane synthase) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). These genes displayed a stepwise

difference in expression level and were not differentially expressed between any of the groups. Housekeeping gene performance was further characterized using the M-value method with the Genorm software package (medgen.ugent.be/genorm) described by Vandesompele et al. [12].

Statistical analysis

Comparison of subject characteristics

Patient characteristics and questionnaire scores were compared between the two groups using Student's T-tests or Chi-Square tests as appropriate, considering a P value of < 0.05 statistically significant.

Computational Analysis of Microarray Data

Affymetrix raw data (CEL files) were analyzed using the Gene Data Expressionist Pro software package in version 2.0 (Gene Data, Basel, Switzerland). First, CEL files were subjected to a quality control procedure with the Refiner module which permits global chip quality control with detection and masking of outliers and array defects, and fluorescence gradient correction. Data was condensed with the RMA algorithm [13]. Within the Analyst module signals were first normalized to a logarithmic mean of 1×10^4 in all experiments. An N-way ANOVA was then performed to compare the three groups. No threshold was set for the Affymetrix detection P-value here. All genes with an ANOVA P-value of $<5\times10^{-6}$ were selected. An Affymetrix detection P-value threshold of < 0.04 was introduced and T-tests for these genes were done for PPI+ versus healthy controls and PPI- patients versus healthy controls (2 T-tests), requiring a minimum of 20% of all experiments per group to deliver valid values with an Affymetrix Detection P-value of < 0.04, otherwise the probeset was excluded. All genes with a fold change of ≥ 1.4 and a T-test P-value of $< 1\times10^{-4}$ were selected.

Analysis of Low Density Array data

TaqMan® LDA results were analyzed with the SDS 2.2.1 software package using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen [14]. The resulting relative quantities (RQs) were compared between groups using one-way ANOVA with Dunnett's post hoc tests. In comparing RQs a P value of < 0.05 was considered statistically significant and a P value of < 0.10 was considered a trend towards significance.

RESULTS

Subjects

The characteristics of the patients and controls are shown in table 1. A total of fourteen healthy controls underwent endoscopy, four healthy controls were excluded upon finding abnormalities (2 grade A esophagitis, 1 grade B esophagitis, 1 Barrett's epithelium).

Table 1. Subject characteristics and symptom scores

	N	m	age	acid exposure(%)	esophagitis (N)	symptoms (all)†	heartburn, pain‡	belching, regurgitation
HC	10	5	49 (37-63)	N/A	0	N/A	N/A	N/A
PPI -	10	6	46 (25-67)	7.1 (5.7 – 9.9)	7	37 (12-57)	21 (4-32)	16 (0-27)
PPI +	10	4	56 (36-75)	9.3 (6.1 – 11.5)	4	21 (0-44)	10 (0-23)	11 (0-23)

Subject characteristics and symptom scores of healthy controls (HC), GERD patients off acid suppressive medication (PPI-), and GERD patients using maximum PPI dose (PPI+).
m: number of male subjects. Age, acid exposure and symptoms are displayed as mean (range).
Symptom scores cover the two weeks prior to endoscopy and sample collection. N/A: not applicable.
†: P = 0.042; ‡: P = 0.017

Age and sex did not differ significantly between the healthy controls and both patient groups and between the patient groups. As expected, PPI- patients had more symptoms in total and especially more heartburn and retrosternal pain than the PPI+ group during the two weeks prior to biopsy collection. Regurgitation and belching, symptoms that are not treated by acid suppression, were equally prevalent in both patient groups. Esophagitis prevalence did not differ significantly between the PPI- and PPI+ groups.

Differentially expressed genes

Microarray mRNA expression profiling

The results from the microarray mRNA expression profiling are summarized in table 2. The raw data are available as CEL files online at the NCBI Gene Expression Omnibus (GEO) page. In patients off PPI, 30 probe sets were upregulated and 23 probe sets were downregulated relative to healthy controls. In patients on PPI, 42 probe sets were upregulated and 5 probe sets were downregulated relative to healthy controls.

Table 2.

Upregulated genes

Gene name	Function Description	Probe Set	***PPI-***	***PPI+***
AGPAT2	1-acylglycerol-3-phosphate O-acyltransferase 2	210678_s_at	2,15	2,32
		32837_at	2,23	2,44
ALDOB	aldolase B, fructose-bisphosphate	214424_s_at		1,61
ALPI	intestinal alkaline phosphatase	211618_s_at	2,03	
		207140_at	1,43	
ASAHL	N-acylsphingosine-amidohydrolase-like	214765_s_at	1,63	
C11orf2	chromosome 11 open reading frame2	217969_at		1,44
C6orf166	chromosome 6 open reading frame 166	223144_s_at		1,45
COMTD1	catechol-O-methyltransferase domain containing 1	226870_at		1,54
CYB5R3	diaphorase (NADH) (cytochrome b-5 reductase)	1554574_a_at	1,41	1,47
EIF5A EIF5AL1 EIF5AL3	eukaryotic translation initiation factor 5A eukaryotic translation initiation factor 5A-like 1 eukaryotic translation initiation factor 5A-like 3	201123_s_at	1,84	1,85
FADD	Fas (TNFRSF6)-associated via death domain	202535_at		1,54
H2AFY	H2A histone family, member Y	214500_at	1,47	1,47
HEXB	hexosaminidase B (beta polypeptide)	201944_at	1,59	1,81
HMGN1	high-mobility group nucleosome binding domain 1	200944_s_at		1,54
HMGN1 ACTN1	high-mobility group nucleosome binding domain 1 actinin, alpha 1	200943_at		1,60
HOOK2	hook homolog 2 (Drosophila)	218780_at		1,52
HSPB1	heat shock 27kDa protein 1	201841_s_at	2,36	2,60
JTB RAB13	jumping translocation breakpoint RAB13, member RAS oncogene family	200048_s_at	1,60	1,64
		210434_x_at	1,52	1,57
		210927_x_at	1,56	1,61
LOC202781	Homo sapiens hypothetical protein LOC202781	235587_at		1,70
LSR	lipolysis stimulated lipoprotein receptor	208190_s_at	2,01	2,11

MED16	mediator complex subunit 16	221938_x_at	1,62	1,75
		43544_at	2,19	2,46
MEN1	multiple endocrine neoplasia I	202645_s_at		1,40
MPZL1	myelin protein zero-like 1	210087_s_at	1,56	1,63
		210594_x_at	1,48	1,59
NCBP2 LOC152217	nuclear cap binding protein subunit 2, 20kDa hypothetical protein BC007882	225657_at	2,00	2,28
PFN1	profilin 1	200634_at	1,58	1,59
PGLS	6-phosphogluconolactonase	218387_s_at	1,66	1,76
		218388_at	1,72	1,77
PGRMC1	progesterone receptor membrane component 1	201120_s_at	1,69	2,01
PPAP2A	phosphatidic acid phosphatase type 2A	209147_s_at	1,63	1,79
		210946_at		1,58
RANBP1	RAN binding protein 1	202483_s_at	1,66	2,00
RNASET2	ribonuclease T2	217983_s_at		2,14
		217984_at		1,53
RNF126	ring finger protein 126	223332_x_at		1,51
SNRP70	small nuclear ribonucleoprotein 70kDa polypeptide	201221_s_at	1,77	1,99
SURF1	surfeit 1	204295_at		1,44
TMEM125	transmembrane protein 125	225822_at	1,50	
TMEM55B	transmembrane protein 55B	225287_s_at	1,51	1,67
UBE2D2	ubiquitin-conjugating enzyme E2D 2	201345_s_at	1,47	
VAPA	VAMP-associated protein A, 33kDa	208780_x_at		1,46
VIPR1	vasoactive intestinal peptide receptor 1	205019_s_at		1,57
YKT6	SNARE protein Ykt6	217785_s_at	1,86	2,00

Downregulated genes

Gene name	Function Description	Probe Set	***PPI-***	***PPI+***
C10orf119	chromosome 10 open reading frame 119	222464_s_at	-1,75	
C14orf129	chromosome 14 open reading frame 129	223239_at	-1,71	
C4orf29	chromosome 4 open reading frame 29	236240_at	-1,56	
CASP8AP2	CASP8 associated protein 2	222201_s_at	-1,57	
CASZ1	castor zinc finger 1	243386_at	-1,52	
CCDC47	coiled-coil domain containing 47	217814_at	-1,63	
CPEB4	cytoplasmic polyadenylation element binding protein 4	224831_at		-1,40
DAPK1	death-associated protein kinase 1	203139_at	-1,83	-1,59
DEGS1	degenerative spermatocyte homolog, lipid desaturase (Drosophila)	209250_at	-1,50	
DMXL1	Dmx-like 1	203791_at	-1,41	
FAM135A	family with sequence similarity 135, member A	223497_at	-1,54	
HACE1	HECT domain and ankyrin repeat containing, E3 ubiquitin protein ligase 1	227471_at	-1,65	

Gene name	Function Description	Probe Set	***PPI-***	***PPI+***
HIBADH	3-hydroxyisobutyrate dehydrogenase	224812_at	-1,64	-1,64
		231955_s_at	-1,44	
ITSN1	intersectin 1 (SH3 domain protein)	209298_s_at	-1,54	
KIAA1468	KIAA1468	225508_at	-1,40	
MAP3K2	mitogen-activated protein kinase kinase kinase 2	221695_s_at	-1,53	
MLX	MAX-like protein X	213708_s_at	-1,57	-1,48
PPTC7	PTC7 protein phosphatase homolog (S. cerevisiae)	225213_at	-1,42	
RASSF6	Transcribed sequences	229147_at	-1,93	
S100A6	S100 calcium binding protein A6 (calcyclin)	217728_at		-1,58
SAMD8	sterile alpha motif domain containing 8	225950_at	-1,62	
SEPSECS	Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase	227982_at	-1,44	
SSFA2	sperm specific antigen 2	202506_at	-1,60	
VAMP2	vesicle-associated membrane protein 2 (synaptobrevin 2)	214792_x_at	-1,42	

Upregulated and downregulated genes in duodenal biopsies from GERD patients off proton pump inhibitors (PPI-) and GERD patients on proton pump inhibitors (PPI+).
Results are arranged with the gene names in alphabetical order. Grey and white lines belong to different sets of results: either one gene was recognized by multiple probe sets, e.g. AKAP1, or one probe set could have recognized more than one gene, e.g. ATRX/LOC728849. In the case of ATP5L/UBE4A, two genes could both have been recognized by three probesets.

TaqMan® Low Density Array validation

The results of the RT-PCR validation experiments are depicted as box plots of relative quantities of transcript in figure 1. Eleven of the 21 selected genes were expressed significantly different, all in the PPI- group.

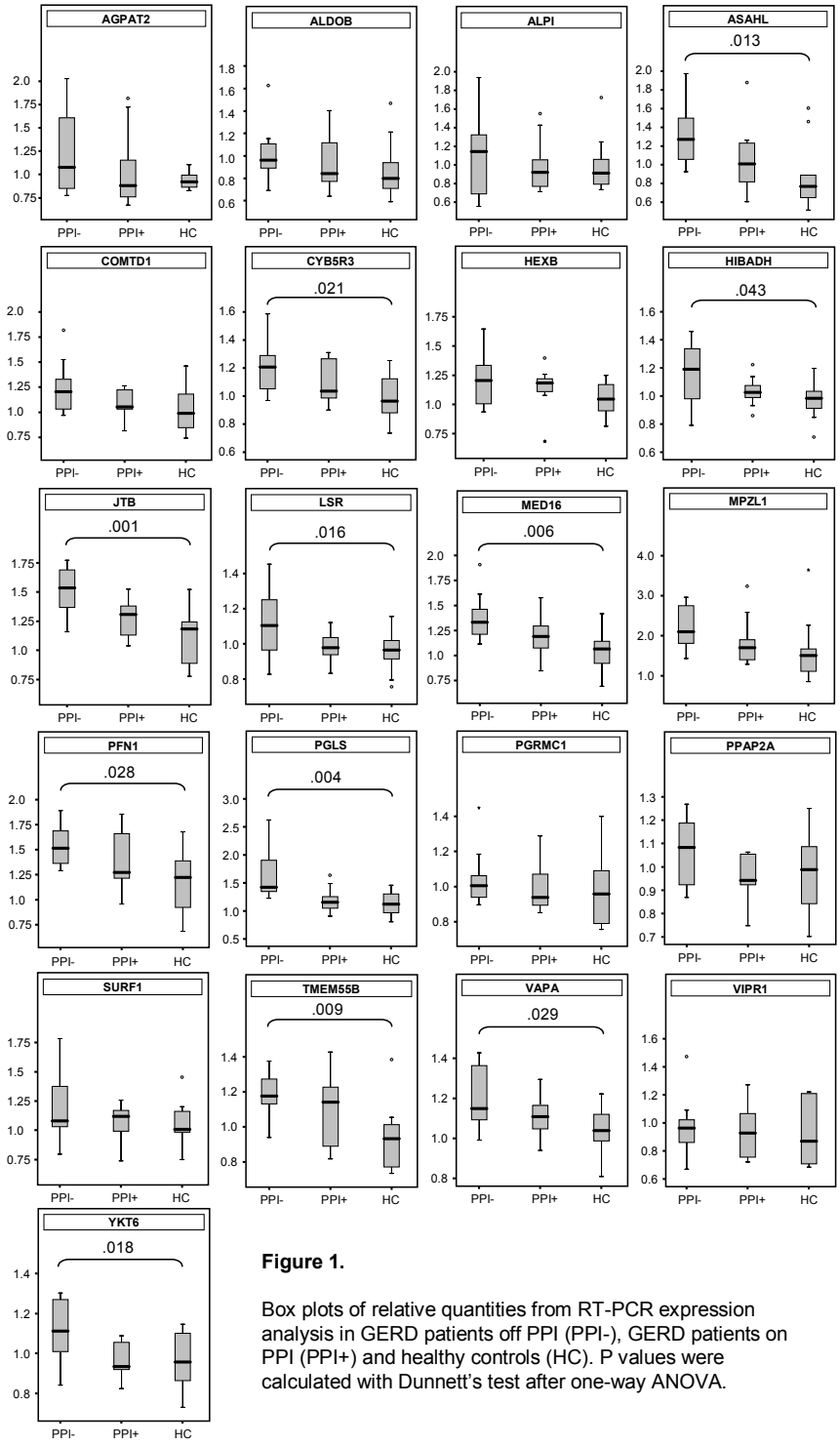


Figure 1.

Box plots of relative quantities from RT-PCR expression analysis in GERD patients off PPI (PPI-), GERD patients on PPI (PPI+) and healthy controls (HC). P values were calculated with Dunnett's test after one-way ANOVA.

DISCUSSION

The current study describes for the first time differences in duodenal mucosal gene expression between GERD patients and control subjects. The genes with non-generic functions could be tied to uptake of lipids, endocytosis, fatty acid metabolism and triglyceride resynthesis, chylomicron formation, vesicle trafficking and exocytosis.

Lipid absorption, fatty acid metabolism, triglyceride resynthesis, packaging in chylomicrons and transport from ER to Golgi in vesicles and to the basolateral space through exocytosis rank among the most basic functions of the small intestine. How can we explain the apparent enhancement of these processes in symptomatic GERD patients? To answer this question, we must look more closely into the composition and function of the chylomicrons that are produced as a result of the upregulation of the found genes. Figure 2 is a schematic representation of the aforementioned processes, expanded to the events taking place in the basolateral space.

The intestine secretes several different lipoproteins: chylomicrons and very-low density lipoproteins are the major ones. The major lipid components of chylomicrons are triglyceride, cholesterol ester, free cholesterol, and phospholipid. Triglyceride is synthesized in the smooth ER and is transferred into the smooth ER lumen. The chylomicron membrane is a mosaic of a small amount of protein, free cholesterol, and saturated triglyceride in a monolayer of phospholipid. Triglycerides are assembled in the core of the chylomicron, which is stabilized with the monolayer surface of amphipathic molecules [15].

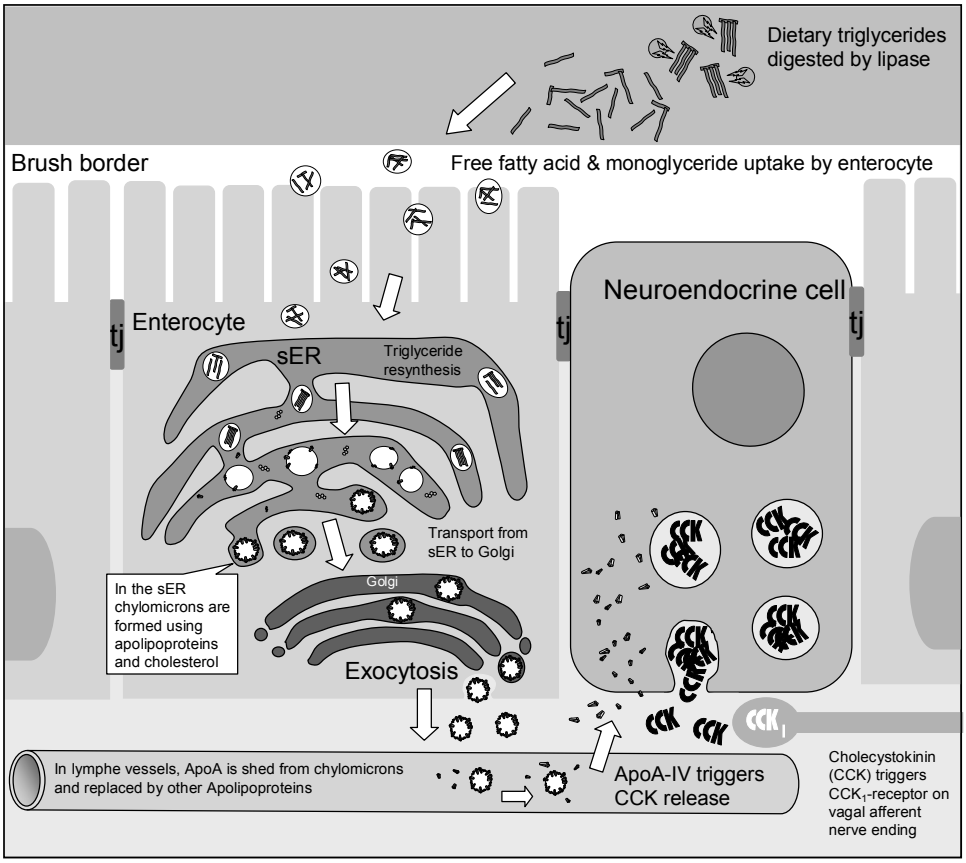
Chylomicrons synthesized in the intestine contain several different apolipoproteins. ApoA-IV comprises 10-13% of chylomicron apolipoproteins. Lipid-carrying prechylomicron transport vesicles (PCTVs) bud from the ER. Subsequently, the PCTVs fuse with the *cis*-Golgi. In the Golgi complex the vesicles are completed for exocytosis from the basolateral membrane [15]. Notably, the transport of chylomicrons from the ER to the Golgi is the rate-limiting step in the process of intestinal lipid absorption [16].

The intestinal lipoproteins do not enter the blood stream directly. Instead they are secreted into tiny lymph vessels that are found inside each of the intestinal villi. Once the intestinal lipoproteins are in contact with other plasma lipoproteins, rapid transfer of proteins occurs.

The loss of chylomicron apolipoproteins is selective, apoA are lost completely and the apolipoproteins left in the chylomicron remnants are apoB, apoE, and apoC. Slower rates of gastric emptying have been reported in GERD patients. Inhibition of gastric emptying by lipid is induced only by fatty acids of chain length greater than C10, so-called long-chain fatty acids. These fatty acids require chylomicron formation for absorption.

Figure 2.

Schematic representation of processes in which the differentially expressed genes play a role.



The genes that were differentially expressed in the duodenum of GERD patients have roles in the processes that are depicted here: fatty acid and monoglyceride uptake through endocytosis, triglyceride resynthesis, chylomicron assembly, sER to Golgi vesicular transport (which is a rate-limiting step in chylomicron synthesis), exocytosis and ApoA-IV production and release. tj: tight junction.

Apolipoprotein A-IV is a component of chylomicrons and active lipid absorption results in apoA-IV release from enterocytes. It has been hypothesized that apoA-IV acts on adjacent CCK-expressing endocrine cells to stimulate release of CCK. CCK then activates CCK_1 receptors on duodenal vagal afferent terminals, thereby initiating feedback inhibition of gastric motility [17]. This hypothesis is supported by the findings that inhibition of gastric emptying in response to intestinal lipid by activation of the vagal afferent pathway via a

CCK_1 receptor-dependent mechanism requires chylomicron formation [18], and expression of apoA-IV [19].

Apolipoprotein A-IV, released from chylomicrons, stimulates duodenal vagal afferent activity to inhibit gastric motility via a CCK_1 pathway. In GERD patients this sensory transduction pathway may be augmented, as well as other sensory pathways which have yet to be clarified.

The genes that were found with the microarrays provide some support for this hypothesis. Increased release of chylomicrons necessitates increased uptake capacity of lipoproteins. LSR, also known as LISCH7, codes for the lipolysis-stimulated lipoprotein receptor, a protein which stimulates uptake of chylomicrons, very low density lipoproteins and high-density lipoproteins in the presence of free fatty acids [20]. The release of CCK has effects on all adjacent cells; the upregulated gene HSPB1 codes for 27 kDa heat shock protein Hsp27, which has a role in stress resistance and a specific role in protecting the actin cytoskeleton against high concentrations of CCK [21].

Several of the found genes have protective roles in the case of cellular stress, which can be heightened in the event of enhanced signal transduction pathways. Cellular stress eventually causes small intestinal cells to either shed from the monolayer or enter apoptotic sequences [22]. The downregulated gene RASSF6 induces apoptosis when abundant [23]. JTB is anti-apoptotic and retards cell growth [24]. MED16, also known as THRAP5, codes for a protein which is part of the Mediator Complex. MED16 is a coactivator of lipopolysacharide-induced transcriptional activators [25] and could have a function in cellular stress response. C14orf129, also known as GSKIP, is a negative regulator of GSK3-beta, which is a part of the Wnt-signaling pathway [26]. Downregulation of GSKIP could therefore promote cell survival and proliferation. DAPK-1 codes for a pro-apoptotic protein; downregulation could have a anti-apoptotic effect [27].

The uptake of lipids and the subsequent metabolism of fatty acids and resynthesis of triglycerides require large amounts of ATP. SURF1 is a part of cytochrome C oxidase, which generates electropotential at the mitochondrion, needed to produce ATP [28]. PGLS catalyses the second step of the pentose-phosphate pathway, resulting in NADPH-production, large amounts of which are needed in triglyceride-resynthesis [29].

Aldolase B (ALDOB) is the glycolytic enzyme that catalyzes the hydrolysis of fructose-1,6-biphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone-3-phosphate, a substrate in the glycerolipid synthesis. DEGS1 codes for a protein with fatty acid desaturase activity [30]. HEXB plays a role in sphingolipid metabolism [31]. HIBADH codes for 3-hydroxyisobutyrate dehydrogenase, which catalyses the breakdown of the 3-hydroxy isoform of butyric acid,

one of the most abundant short chain fatty acids, and is a part of triglyceride resynthesis pathways [32]. Cytochrome B5 reductase (CYB5R3) catalyses the desaturation and elongation of fatty acids, and also has a role in cholesterol biosynthesis. ASAHL degrades fatty acid amides to free fatty acids [33]. VIPR1 codes for the vasoactive intestinal peptide (VIP) receptor 1. VIP stimulates long chain fatty acid oxidation [34]. PPAP2A catalyses the dephosphorylation of phosphatidic acid (PA) to form diacylglycerol (DG). There is evidence that PPAP2A is located at the plasma membrane where it plays an active role in the hydrolysis and uptake of lipids from the extracellular space [35]. AGPAT2 codes for an enzyme that converts lysophosphatidic acid (LPA) into phosphatidic acid (PA), which is a precursor for triglyceride synthesis. Moreover, PA is an intracellular signal molecule with a role in vesicle transport and budding [36].

PGMRC1 codes for a transmembrane receptor for several steroids, it localizes to the endoplasmatic reticulum (ER) where it may have a role in the biosynthesis of cholesterol [37] which is a key component of all lipoproteins. MPZL1, also known as PZR, codes for a transmembrane protein which specifically binds Src homology protein 2, which influences serum ApoB and LDL cholesterol levels [38].

Fat absorption induces endocytosis via clathrin-coated pits from the enterocyte brush border; this process selectively internalizes intestinal alkaline phosphatase, encoded by the ALPI gene [39]. PFN1 or profilin 1 binds 1:1 to actin monomers and is essential for vesicle trafficking at the trans-Golgi network [40]. TMEM55B encodes a 4-phosphatase that catalyzes the hydrolysis of the signaling molecule phosphatidylinositol-4,5-biphosphate (PtdIns-4,5-P_2) to phosphatidylinositol-5-phosphate (PtdIns-5-P). PtdIns-5-P influences the filamentous actin cytoskeleton, essential for vesicle trafficking [41]. VAMP2 or synaptobrevin 2 is essential for exocytosis [42]. VAPA or VAMP-A codes for vesicle-associated membrane protein associated-A, which has a role in ER to Golgi vesicle transport [43]. YKT6 plays an essential and central role in the secretory pathway, foremost in ER to Golgi transport [44]. ITSN1 plays a role in endocytosis, vesicle trafficking and exocytosis [45, 46]. RAB13 codes for a small GTPase which is a component of the junctional complex of polarized epithelial cells, and which is a specific regulator of vesicle trafficking [47].

RT-PCR analyses confirmed deregulation of the majority of the selected genes, practical and financial limits barred confirmation of all genes. One can assume, however, that the majority of the up and downregulated genes discerned with the use of microarrays reflects reality.

Aside from VIP, with its hormonal effects on fluid and electrolyte secretion, two other genes with effects on secretion were deregulated. S100A6 is involved in secretion by mucosal cells

in the intestine, mainly goblet cells, this gene was downregulated in PPI+ patients [48]. Catechol-O-methyltransferase domain containing protein 1 (COMTD1) has the ability to degrade catecholamines with the COMT-domain. COMT upregulation results in a drop in bicarbonate secretion [49].

The differences between gene expression in the PPI- and PPI+ patient groups support the hypothesis regarding enhanced duodenal afferent feedback, since the PPI- group experienced two weeks of unabated heartburn symptoms.

In conclusion, the present study describes for the first time gene expression differences in the duodenal mucosa between patients with GERD on and off PPI and healthy controls. The gene expression differences that were found suggest that in symptomatic GERD patients uptake and metabolism of fatty acids, triglyceride resynthesis, chylomicron formation and transport and possibly signaling by ApoA-IV and CCK are enhanced.

References

1. Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus 1. Am J Gastroenterol 2006; 101(8):1900-1920.

2. Camilleri M, Dubois D, Coulie B et al. Prevalence and socioeconomic impact of upper gastrointestinal disorders in the United States: results of the US Upper Gastrointestinal Study. Clin Gastroenterol Hepatol 2005; 3(6):543-552.

3. Dent J, El Serag HB, Wallander MA, Johansson S. Epidemiology of gastrooesophageal reflux disease: A systematic review. Gut 2005; 54(5):710-717.

4. Holloway RH, Lyrenas E, Ireland A, Dent J. Effect of intraduodenal fat on lower oesophageal sphincter function and gastro-oesophageal reflux. Gut 1997; 40(4):449-453.

5. Ledeboer M, Masclee AA, Biemond I, Lamers CB. Effect of medium- and long-chain triglycerides on lower esophageal sphincter pressure: role of CCK. Am J Physiol 1998; 274(6 Pt 1):G1160-G1165.

6. Meyer JH, Lembo A, Elashoff JD, Fass R, Mayer EA. Duodenal fat intensifies the perception of heartburn. Gut 2001; 49(5):624-628.

7. De Vries DR, Van Herwaarden MA, Baron A, Smout A, Samsom M. Concomitant functional dyspepsia and irritable bowel syndrome decrease health-related quality of life in gastroesophageal reflux disease. Scand J Gastroenterol 2007; 42(8):951-956.

8. Barbera R, Feinle C, Read NW. Nutrient-specific modulation of gastric mechanosensitivity in patients with functional dyspepsia. Dig Dis Sci 1995; 40(8):1636-1641.

9. Sarkar S, Thompson DG, Woolf CJ, Hobson AR, Millane T, Aziz Q. Patients with chest pain and occult gastroesophageal reflux demonstrate visceral pain hypersensitivity which may be partially responsive to acid suppression. Am J Gastroenterol 2004; 99(10):1998-2006.

10. Trimble KC, Farouk R, Pryde A, Douglas S, Heading RC. Heightened visceral sensation in functional gastrointestinal disease is not site-specific. Evidence for a generalized disorder of gut sensitivity. Dig Dis Sci 1995; 40(8):1607-1613.

11. Talley NJ, Verlinden M, Jones M. Validity of a new quality of life scale for functional dyspepsia: a United States multicenter trial of the Nepean Dyspepsia Index. American Journal of Gastroenterology 1999; 94(9):2390-2397.

12. Vandesompele J, De PK, Pattyn F et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3(7):RESEARCH0034.

13. Irizarry RA, Hobbs B, Collin F et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostat 2003; 4(2):249-264.

14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method 1. Methods 2001; 25(4):402-408.

15. Black DD. Development and physiological regulation of intestinal lipid absorption. I. Development of intestinal lipid absorption: cellular events in chylomicron assembly and secretion. Am J Physiol Gastrointest Liver Physiol 2007; 293(3):G519-G524.

16. Mansbach CM, Nevin P. Intracellular movement of triacylglycerols in the intestine. J Lipid Res 1998; 39(5):963-968.

17. Raybould HE, Glatzle J, Freeman SL et al. Detection of macronutrients in the intestinal wall. Auton Neurosci 2006; 125(1-2):28-33.

18. Glatzle J, Kalogeris TJ, Zittel TT, Guerrini S, Tso P, Raybould HE. Chylomicron components mediate intestinal lipid-induced inhibition of gastric motor function. Am J Physiol Gastrointest Liver Physiol 2002; 282(1):G86-G91.

19. Whited KL, Lu D, Tso P, Kent Lloyd KC, Raybould HE. Apolipoprotein A-IV is involved in detection of lipid in the rat intestine. J Physiol 2005; 569(Pt 3):949-958.

20. Yen FT, Roitel O, Bonnard L et al. Lipolysis Stimulated Lipoprotein Receptor: a novel molecular link between hyperlipidemia, weight gain, and atherosclerosis in mice. J Biol Chem 2008; 283(37):25650-25659.

21. Schafer C, Clapp P, Welsh MJ, Benndorf R, Williams JA. HSP27 expression regulates CCK-induced changes of the actin cytoskeleton in CHO-CCK-A cells. Am J Physiol 1999; 277(6 Pt 1):C1032-C1043.

22. Hotchkiss RS, Schmieg RE, Jr., Swanson PE et al. Rapid onset of intestinal epithelial and lymphocyte apoptotic cell death in patients with trauma and shock. Crit Care Med 2000; 28(9):3207-3217.

23. Ikeda M, Hirabayashi S, Fujiwara N et al. Ras-association domain family protein 6 induces apoptosis via both caspase-dependent and caspase-independent pathways. Exp Cell Res 2007; 313(7):1484-1495.

24. Kanome T, Itoh N, Ishikawa F et al. Characterization of Jumping translocation breakpoint (JTB) gene product isolated as a TGF-beta1-inducible clone involved in regulation of mitochondrial function, cell growth and cell death. Oncogene 2007; 26(41):5991-6001.

25. Kim TW, Kwon YJ, Kim JM, Song YH, Kim SN, Kim YJ. MED16 and MED23 of Mediator are coactivators of lipopolysaccharide- and heat-shock-induced transcriptional activators. Proc Natl Acad Sci U S A 2004; 101(33):12153-12158.

26. Chou HY, Howng SL, Cheng TS et al. GSKIP is homologous to the Axin GSK3beta interaction domain and functions as a negative regulator of GSK3beta. Biochemistry 2006; 45(38):11379-11389.

27. Bialik S, Kimchi A. The death-associated protein kinases: structure, function, and beyond. Annu Rev Biochem 2006; 75:189-210.

28. Tiranti V, Galimberti C, Nijtmans L, Bovolenta S, Perini MP, Zeviani M. Characterization of SURF-1 expression and Surf-1p function in normal and disease conditions. Hum Mol Genet 1999; 8(13):2533-2540.

29. Collard F, Collet JF, Gerin I, Veiga-da-Cunha M, Van SE. Identification of the cDNA encoding human 6-phosphogluconolactonase, the enzyme catalyzing the second step of the pentose phosphate pathway(1). FEBS Lett 1999; 459(2):223-226.

30. Cadena DL, Kurten RC, Gill GN. The product of the MLD gene is a member of the membrane fatty acid desaturase family: overexpression of MLD inhibits EGF receptor biosynthesis. Biochemistry 1997; 36(23):6960-6967.

31. Sandhoff K, Kolter T. Processing of sphingolipid activator proteins and the topology of lysosomal digestion. Acta Biochim Pol 1998; 45(2):373-384.

32. Kedishvili NY, Popov KM, Jaskiewicz JA, Harris RA. Coordinated expression of valine catabolic enzymes during adipogenesis: analysis of activity, mRNA, protein levels, and metabolic consequences. Arch Biochem Biophys 1994; 315(2):317-322.

33. Tsuboi K, Sun YX, Okamoto Y, Araki N, Tonai T, Ueda N. Molecular characterization of N-acylethanolamine-hydrolyzing acid amidase, a novel member of the choloylglycine hydrolase family with structural and functional similarity to acid ceramidase. J Biol Chem 2005; 280(12):11082-11092.

34. Vidal H, Beylot M, Comte B, Vega F, Riou JP. Vasoactive intestinal peptide stimulates long-chain fatty acid oxidation and inhibits acetyl-coenzyme A carboxylase activity in isolated rat enterocytes. J Biol Chem 1989; 264(9):4901-4906.

35. Roberts RZ, Morris AJ. Role of phosphatidic acid phosphatase 2a in uptake of extracellular lipid phosphate mediators. Biochim Biophys Acta 2000; 1487(1):33-49.

36. Jones D, Morgan C, Cockcroft S. Phospholipase D and membrane traffic. Potential roles in regulated exocytosis, membrane delivery and vesicle budding. Biochim Biophys Acta 1999; 1439(2):229-244.

37. Losel RM, Besong D, Peluso JJ, Wehling M. Progesterone receptor membrane component 1--many tasks for a versatile protein. Steroids 2008; 73(9-10):929-934.

38. Jamshidi Y, Gooljar SB, Snieder H et al. SHP-2 and PI3-kinase genes PTPN11 and PIK3R1 may influence serum apoB and LDL cholesterol levels in normal women. Atherosclerosis 2007; 194(2):e26-e33.

39. Hansen GH, Niels-Christiansen LL, Immerdal L, Nystrom BT, Danielsen EM. Intestinal alkaline phosphatase: selective endocytosis from the enterocyte brush border during fat absorption. Am J Physiol Gastrointest Liver Physiol 2007; 293(6):G1325-G1332.

40. Dong J, Radau B, Otto A, Muller E, Lindschau C, Westermann P. Profilin I attached to the Golgi is required for the formation of constitutive transport vesicles at the trans-Golgi network. Biochim Biophys Acta 2000; 1497(2):253-260.

41. Sbrissa D, Ikonomov OC, Strakova J, Shisheva A. Role for a novel signaling intermediate, phosphatidylinositol 5-phosphate, in insulin-regulated F-actin stress fiber breakdown and GLUT4 translocation. Endocrinology 2004; 145(11):4853-4865.

42. Lang T, Jahn R. Core proteins of the secretory machinery. Handb Exp Pharmacol 2008;(184):107-127.

43. Lotz GP, Brychzy A, Heinz S, Obermann WM. A novel HSP90 chaperone complex regulates intracellular vesicle transport. J Cell Sci 2008; 121(Pt 5):717-723.

44. Fukasawa M, Varlamov O, Eng WS, Sollner TH, Rothman JE. Localization and activity of the SNARE Ykt6 determined by its regulatory domain and palmitoylation. Proc Natl Acad Sci U S A 2004; 101(14):4815-4820.

45. Wang W, Bouhours M, Gracheva EO et al. ITSN-1 controls vesicle recycling at the neuromuscular junction and functions in parallel with DAB-1. Traffic 2008; 9(5):742-754.

46. Yu Y, Chu PY, Bowser DN et al. Mice deficient for the chromosome 21 ortholog Itsn1 exhibit vesicle trafficking abnormalities. Hum Mol Genet 2008.

47. Nokes RL, Fields IC, Collins RN, Folsch H. Rab13 regulates membrane trafficking between TGN and recycling endosomes in polarized epithelial cells. J Cell Biol 2008; 182(5):845-853.

48. Timmons PM, Chan CT, Rigby PW, Poirier F. The gene encoding the calcium binding protein calcyclin is expressed at sites of exocytosis in the mouse. J Cell Sci 1993; 104 (Pt 1):187-196.

49. Knutson L, Knutson TW, Flemstrom G. Endogenous dopamine and duodenal bicarbonate secretion in humans. Gastroenterology 1993; 104(5):1409-1413.

SUMMARY

Chapter 1 offers a general introduction to the pathophysiology of gastroesopheageal reflux disease (GERD). The mechanisms through which a physiological phenomenon like gastroesophageal reflux can cause the disease GERD are reviewed. An overview of the differential diagnosis which can be clarified with the essential diagnostic tools esophageal manometry and 24-hour esophageal pH-metry is given. The possibilities for treatment are reviewed and placed in perspective.
GERD symptoms and complications cluster in families, and twin siblings of GERD patients have an increased risk of developing GERD as well. This hereditary tendency towards developing GERD could be explained by variations or polymorphisms in the genetic code. One such polymorphism, GNB3 825 C/T, is used as an example of how minute changes in the DNA can lead to altered functionality of a protein, chapter 6 contains the result of an association study involving this polymorphism. A brief explanation of genome-wide RNA expression profiling is provided as a prelude to chapters 8 and 9. The introduction concludes with the definition of translational medicine.

Several studies have shown that gastrointestinal symptoms tend to cluster in the general population; a subject suffering from typical GERD symptoms runs a higher risk of having symptoms of functional dyspepsia (FD) and/or irritable bowel syndrome (IBS) as well. The prevalence of concomitant FD and IBS symptoms in patients whose GERD has been proven with 24-hour pH-metry, however, had not been investigated yet.
In **chapter 2** the prevalence of functional dyspepsia (FD) and irritable bowel syndrome (IBS) in a large group of GERD patients is investigated. Among the 263 included patients with GERD that had been diagnosed with 24-hour pH-metry the prevalence was higher than in the general population: 25% had FD and 35% had IBS. These concomitant symptoms had a significant impact on health-related quality of life; patients with only GERD symptoms and no concomitant FD and/or IBS had a quality of life score that was only slightly lower than in healthy controls.

Numerous studies link obesity to GERD. The impact of a high body mass index (BMI) on parameters with a role in GERD pathogenesis has been studied extensively as well. However, studies integrating GERD patients' age and BMI with data collected during

esophageal manometry, 24-hour intraesophageal pH-monitoring and upper endoscopy were lacking.

In **chapter 3** the results from esophageal manometry, 24-hour pH-metry and upper GI endoscopy in 149 GERD patients were investigated and correlated to age and BMI using multivariate analyses. 103 patients had a hiatus hernia. BMI predicted intragastric pressure, the gastroesophageal pressure gradient during inspiration and intraesopahegeal pressure during expiration. Age predicted intraesophageal pressure and the gastroesophageal pressure gradient during inspiration. Presence of hiatus hernia was predicted by intragastric pressure, the gastroesophageal pressure gradient and BMI. The gastroesophageal pressure gradient, however, did not show an association with esophageal acid exposure during 24-hour pH-metry.

Some patients consult their physician complaining about typical GERD symptoms, but when 24-hour pH-metry is performed, acid exposure to the esophagus turns out to be well within the physiological range. With the use of symptom association scores like the symptom index (SI) and symptom association probability (SAP) a large number of these patients can prove that they are hypersensitive to acid and feel the reflux too accurately. No studies were published comparing groups of patients with physiological reflux but positive symptom association scores to GERD patients with pathological reflux with respect to demographic data and efficacy of medical treatment, symptom severity, concomitant symptoms of FD and IBS and health-related quality of life.

In **chapter 4** the differences between GERD patients with physiological acid exposure during 24-hour pH-metry (<6% total time with pH <4) but positive SI and/or SAP and GERD patients with pathological acid exposure are explored. The patients with physiological acid exposure turned out to be younger, more often females, and scoring worse symptoms which reacted to PPIs less often. In addition the prevalence of FD and IBS, which had already been shown to be higher in chapter 1, was significantly higher in this GERD subgroup. As could be expected, the prevalence of esophagitis and hiatus hernia was lower in the group with physiological acid exposure, however, their health-related quality of life was lower in several domains.

Proton pump inhibitors (PPIs) are the cornerstone of GERD treatment today. Almost all PPIs are metabolized by cytochrome P450 2C19 (CYP2C19). Failure of PPI treatment is the main reason for patients to undergo antireflux surgery.

Chapter 5 explores the possible impact of a gene polymorphism (CYP2C19*2), which influences PPI metabolism and efficacy, on referral of GERD patients for surgical therapy. In this group no association was found between PPI metabolism, reflected by CYP2C19*2 genotype, and referral for surgery.

Candidate genes for associations with GERD could be located in signal transduction cascades. The G-protein coupled receptors (GPCRs) form an enormous family of proteins that have seven transmembrane domains, to which the heterotrimeric G-protein is coupled, as a common feature. The majority of signaling cascades contain one or more GPCRs, therefore a functional genetic polymorphism in a G-protein subunit would make a valid candidate for association studies concerning GERD.
In **chapter 6** a single nucleotide polymorphism in the G protein beta-3 subunit (GNB3 825 C/T) is investigated. This SNP causes enhanced second messenger signal transduction upon activation of numerous G-protein-coupled receptors. Carriers of the T allele were overrepresented in GERD patients relative to healthy controls. The association was stronger in the subgroups that were characterized by physiological acid exposure, a positive symptom association score and in the group that had no concomitant FD and/or IBS.

The neurotransmitter 5-hydroxytryptamine (5-HT) or serotonin plays a major role in intestinal sensitivity and motility. Genetic polymorphisms in the serotonergic signaling pathway would be valid candidate genes for associations with GERD as well.
In **chapter 7** three genetic polymorphisms in genes of 5-HT signaling pathway are determined in large groups of GERD patients and healthy controls. The insertion/deletion polymorphism in the 5-HT transporter promoter region (SERT-P), a polymorphism in the upstream open reading frame of the 5-HT_3 receptor subunit A (HTR3A) and a single nucleotide polymorphism in the promoter region of tryptophan hydroxylase 2, an enzyme catalyzing the rate-limiting step of 5-HT synthesis which is predominantly expressed in the central nervous system, were genotyped. In the investigated group no associations were found of any single polymorphism with GERD. However, the combined carriership of the T-allele of the HTR3A SNP and homozygosity for the long SERT-P variant was more prevalent in patients with pathological reflux, and markedly more prevalent in patients with a hypotensive lower esophageal sphincter.

All changes in cells and tissues are accompanied by changes in RNA transcription, also known as gene expression. The innate qualities and the reactions to acid reflux in the

esophageal epithelium of GERD patients, relative to healthy controls, could cause differences in gene expression reflected by RNA content of tissue samples. No studies using genome-wide expression analysis of biopsy samples of thoroughly phenotyped GERD patients had been done to date. The same holds true for duodenal gene expression differences between GERD patients and healthy volunteers, which could provide clues towards understanding how the duodenum influences GERD symptom generation.

In chapters 8 and 9 RNA expression profiling with microarrays, followed by validation through real-time RT-PCR, was used to explore differences in gene expression between GERD patients and healthy controls. Three groups were formed: 10 GERD patients on PPIs, 10 GERD patients who had not used PPIs for two weeks at the time of sampling and a group of 10 healthy controls.

In **chapter 8** gene expression in esophageal mucosal biopsies was investigated. The genes that were found to be differentially expressed pointed towards a role for a process called epithelial restitution in the first defense of the esophagus against acid reflux. This process is characterized by a rapid restitution of epithelial continuity without the need for proliferation of cells, and is driven by energy-dependent changes in the cytoskeleton and cell-cell contacts. Somewhat surprisingly no expression differences of genes coding for pro-inflammatory or neuro-irritative substances were found. The gene expression differences were less pronounced though not absent in the GERD patients off PPIs.

In **chapter 9** the same techniques were used to investigate gene expression differences in the duodenal mucosa of GERD patients relative to healthy controls. The genes that were differentially expressed had functions in uptake of fatty acids, triglyceride resynthesis, chylomicron formation and vesicle trafficking, several of these genes were rate-limiting in this chain of processes. The results pointed towards an enhanced Apolipoprotein A-IV content and secretion in chylomicrons. ApoA-IV is a signaling molecule that can trigger CCK release from neuroendocrine cells, providing a clue towards the mechanism underlying the influence of the duodenum on GERD symptoms.

Nederlandse samenvatting

In dit proefschrift worden patiënten bestudeerd die lijden aan gastro-oesofageale refluxziekte (GORZ). Hiermee worden alle mensen bedoeld die door het terugstromen van maaginhoud, omhoog de slokdarm in, hinderlijke symptomen ontwikkelen of schade aan de slokdarm oplopen.
De klassieke symptomen van GORZ zijn zuurbranden, pijn achter het borstbeen die niets met het hart te maken heeft en oprispingen, maar GORZ kan ook de oorzaak zijn van chronisch hoesten, heesheid, tandbederf, slechte adem en verergering van astma. Vele mensen in de Westerse landen lijden aan GORZ (tot wel een vijfde van alle mensen heeft wekelijks last) en de zuurremmende medicijnen die ertegen worden voorgeschreven staan al jaren in de top tien van meest voorgeschreven geneesmiddelen.

Omdat GORZ zo veel voorkomt ziet de huisarts het grootste deel van de patiënten, en een groot deel van deze patiënten kan afdoende geholpen worden met een voorschrift van medicijnen. Als deze niet helpen of er zijn alarmerende symptomen kan de huisarts de patiënt verwijzen naar een maag-darm-leverarts. Deze zal in de meeste gevallen een oesofago-gastro-duodenoscopie uitvoeren (meestal afgekort tot gastroscopie). Alarmerende symptomen zijn bijvoorbeeld het niet willen zakken van eten en/of drinken en gewichtsverlies, deze symptomen zijn verdacht voor slokdarmkanker en een gastroscopie moet uitwijzen of hiervan sprake is. Een maag-darm-leverarts kan na de scopie een volgende stap bepalen, bijvoorbeeld meer medicijnen of een verwijzing naar een andere specialist als er meer aan de hand blijkt te zijn dan GORZ.

Wanneer de refluxklachten niet afnemen ondanks de maatregelen van de specialist of de huisarts, kan een 24-uurs zuurmeting van de slokdarm worden gedaan, voorafgaand daaraan wordt meestal een drukmeting van de slokdarm verricht waarmee een aantal ziekten die ook pijn achter het borstbeen en oprispingen kunnen veroorzaken kan worden aangetoond of uitgesloten. Dit onderzoek wordt als het beste diagnostische middel beschouwd in de praktijk en door wetenschappers; GORZ is er namelijk mee uit te sluiten. De slokdarm wordt normaliter niet langer dan 6% van een etmaal aan zuur blootgesteld, wanneer de totale tijd waarin de pH in de slokdarm onder de 4 is langer is dan 6% van de gemeten tijd spreken we van een pathologische zuurexpositie. Tijdens de meting bestaat de mogelijkheid om wanneer een symptoom optreedt op een knop te drukken, het symptoom

kan dan in een dagboekje omschreven worden. De drukken op de knop zijn te zien in de meting, en er kan mee worden uitgerekend of deze symptomen met het binnenstromen van zuur in de slokdarm samenhangen of niet. De scores die hiervoor in ons lab worden gebruikt zijn de symptoomindex (SI; positief bij 50% en hoger) en de symptom association probability (SAP; positief bij 95% en hoger).
Bij de drukmeting of manometrie van de slokdarm die voorafgaat aan de 24-uurs zuurmeting kan de ontspanning en de rustdruk van de onderste slokdarmsfincter (lower esophageal sphincter, LES) worden gemeten, deze rustdruk is normaal 0,6 kPa of hoger. Ook kan uit deze meting worden bepaald of de slokdarmperistaltiek normaal is, of dat de samentrekkingen te zwak of niet gecoördineerd zijn. De druk in de maag wordt ook gemeten, deze speelt in de kliniek geen rol maar wordt wel bestudeerd in **hoofdstuk 3**.

GORZ kan behandeld worden met zuurremmende medicijnen, als deze niet werken kan een patiënt baat hebben bij een fundoplicatie. De verwijzing naar de chirurg voor deze operatie komt aan bod in **hoofdstuk 5**.

Mensen met klachten van het maag-darmkanaal hebben zelden alleen klachten van zuurbranden of alleen klachten van de maag, een combinatie van klachten komt vaak voor. **Hoofdstuk 2** beschrijft een studie waarin gekeken werd naar het voorkomen van klachten die niet bij GORZ passen maar wel bij chronische onverklaarde maagklachten (functionele dyspepsie, FD) en bij het prikkelbare darmsyndroom (irritable bowel syndrome, IBS). Ook werd met vragenlijsten de gezondheidsgerelateerde kwaliteit van leven (KvL) in kaart gebracht. Bij 263 mensen bij wie GORZ was bewezen met een 24-uurs zuurmeting had 25% FD en 35% IBS. Dit is meer dan in de algemene bevolking. De KvL was beduidend verlaagd bij de mensen die behalve GORZ ook FD en/of IBS hadden – sterker nog: mensen met alleen maar GORZ hadden niet eens een veel lagere KvL dan gezonde controlepersonen.

Er zijn vele verbanden gelegd tussen de overgewicht-epidemie en de toename in het aantal GORZ-patiënten, maar hoe dit precies werkt is niet helemaal duidelijk. In **hoofdstuk 3** wordt een aantal vragen beantwoord die hiermee samenhangen. De gegevens verkregen bij 149 patiënten met GORZ werden vergeleken, het gaat om uitkomsten van vragenlijsten, manometrie van de slokdarm, 24-uur zuurmetingen en gastroscopie. Uit dit laatste bleek dat 103 patiënten een middenrifbreuk (hernia diafragmatica, HD) hadden. Uit de slokdarm-manometrieën werden de maagdruk, de slokdarmdruk en het verschil hiertussen, de drukgradiënt, berekend tijdens inademen en uitademen. Om maagzuur de slokdarm in te

laten stromen, moet er een drukverschil, een drukgradiënt zijn tussen de maag en slokdarm. Bij GORZ-patiënten zijn grotere gradiënten beschreven dan bij gezonde controles.
Deze drukparameters werden met multivariate analyse gecorreleerd aan andere maten. De body mass index (BMI) was een voorspeller voor de druk in de maag en voor de drukgradiënt tijdens inademen en de slokdarmdruk tijdens uitademen. Leeftijd was een voorspeller voor slokdarmdruk en drukgradiënt tijdens inademen. Het hebben van een HD werd voorspeld door maagdruk, drukgradiënt en BMI. De drukgradiënt voorspelde echter niet de blootstelling aan zuur tijdens de 24-uursmeting.

Er zijn patiënten die met een typisch verhaal van GORZ bij de maag-darm-leverarts komen, maar die wanneer er een zuurmeting wordt verricht minder dan de pathologische 6% van de tijd een pH onder de 4 hebben in de slokdarm. Deze patiënten hebben regelmatig wel een positieve SI en/of SAP, wat inhoudt dat zij bijna elke keer dat er een beetje zuur in de slokdarm komt dit voelen, en er zelfs onder lijden en hulp vragen aan hun arts. Deze patiënten zijn behalve overgevoelig ook erg interessant voor onderzoekers omdat bij hen de mechanismen waardoor zuur uit de maag het typische beeld van GORZ veroorzaakt niet vertroebeld worden door beschadigingen aan de slokdarm. In **hoofdstuk 4** worden patiënten uit deze categorie vergeleken met patiënten die wel een pathologische hoeveelheid zuur in de slokdarm hadden tijdens de 24-uurs zuurmeting. Het blijkt dat de overgevoelige patiënten vaker vrouw zijn en een stukje jonger, en dat zij hun symptomen ernstiger vinden dan patienten met een pathologische zuurexpositie. Bovendien komen FD en IBS vaker voor bij deze patienten en scoren zij op een aantal onderdelen een lagere kwaliteit van leven. Objectief bezien is bij deze overgevoelige patienten de GORZ juist minder ernstig: er is minder vaak sprake van slokdarmontsteking of een middenrifbreuk.

Er bestaat een erfelijke aanleg voor het ontwikkelen van GORZ. Dit is te merken aan de hogere kans die familieleden van GORZ-patienten en vooral tweelingbroers en –zussen lopen om de ziekte te krijgen. Een aantal kandidaatgenen dat mogelijk een aanleg voor GORZ kan versterken werd door ons onderzocht bij een grote groep GORZ-patiënten en vergeleken met controlepersonen.

In het onderzoek dat beschreven wordt in **hoofdstuk 5** werd onderzocht of een variatie in het CYP2C19-gen, dat codeert voor een eiwit dat de meeste zuurremmende protonpompremmers afbreekt, meer voorkomt bij GORZ-patiënten die behandeld worden met een operatieve ingreep. Omdat de operatie wordt gedaan als medicijnen onvoldoende

werken, zou men zich voor kunnen stellen dat patiënten die de medicijnen door de variatie in het gen sneller afbreken vaker geopereerd moeten worden dan mensen die het medicijn minder snel afbreken. Hiervoor werd echter in de door ons onderzochte groep geen bewijs gevonden.

In **hoofdstuk 6** komt een variatie in het GNB3-gen aan bod. Vele verschillende signalen worden via G-eiwitten, waar GNB3 een onderdeel van is, doorgegeven van extracellulaire receptoren naar intracellulaire signaaltransductiecascades. De genvariant waarbij op plaats 825 een C vervangen is door een T werkt minder selectief en geeft signalen vlugger door dan de oorspronkelijke variant. Als dit ook gebeurt bij mensen die zuur in de slokdarm krijgen, voelen mensen met de T-variant misschien eerder zuurbranden. Inderdaad kwam bij GORZ-patiënten de GNB3 825T variant meer voor, vooral bij mensen die in de meer gevoelige categorieën vallen zoals beschreven in hoofdstukken 2 en 4.

In **hoofdstuk 7** komen drie variaties aan bod van genen die een rol spelen in de stofwisseling van serotonine (5-HT), dat zowel van belang is bij sensibiliteit als motoriek van het maagdarmkanaal. Het betreft een 44 baseparen tellende variatie in de promoter-regio van de serotonine-transporter, SERT-P L/S, die bepaalt of er veel of weinig SERT wordt gemaakt, en aldus indirect of er veel of weinig 5-HT beschikbaar is. Een tweede variant is gelegen in het stroomopwaarts gelegen regulatoire gebied (upstream open reading frame, uORF) van de 5-HT_3-receptor subunit A (HTR3A -178C/T). Deze variatie beinvloedt de translatie van mRNA tot functionele eiwitten. Vermoedelijk komen receptoren die bestaan uit alleen A subunits meer voor bij dragers van de T-variant; deze hebben een lagere affiniteit voor 5-HT en desensitiseren sneller. Een derde genvariant die werd onderzocht is gelegen in het gen TPH2, dat een rol speelt bij de synthese van 5-HT.
Er werd geen associatie gevonden met een van de varianten op zich, maar de combinatie van SERT-L met HTR3A T-dragerschap kwam meer voor bij GORZ-patiënten. Deze combinatie zorgt mogelijk voor minder beschikbaar 5-HT terwijl er meer A-subunits zijn, al met al dus minder 5-HT signaal.

In **hoofdstukken 8 en 9** wordt de genexpressie in biopten verkregen bij GORZ-patiënten vergeleken met de genexpressie in biopten verkregen bij gezonde vrijwilligers. De genexpressie in slokdarmepitheel kan worden beschouwd als weerspiegeling van de reactie op blootstelling aan zuur. De genexpressie in het duodenum van GORZ-patiënten zou

aanwijzingen kunnen opleveren voor mechanismen die ten grondslag liggen aan de invloed van het duodenum op de perceptie van zure reflux.

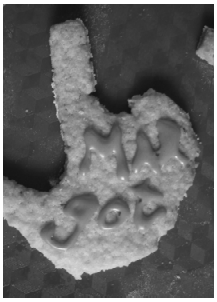
In het onderzoek dat beschreven wordt in **hoofdstuk 8** werden epitheelbiopten uit de slokdarm onderzocht met behulp van microarray expressieanalyse waarna de verschillende genen werden geverifieerd met RT-PCR. Drie groepen van 10 mensen werden vergeleken: een groep GORZ-patiënten die twee weken geen zuurremmende medicijnen gebruikt had, een groep GORZ-patiënten die hun medicijnen had doorgebruikt en een groep gezonde controlepersonen. Uit de vergelijking kwam naar voren dat bij GORZ-patiënten die twee weken ongebreidelde zure reflux hadden gehad de expressie van een aantal genen anders was in vergelijking met gezonde controles. Deze genen hadden bijna allemaal te maken met een proces dat eerder in de darm en maag beschreven is en epitheliale restitutie wordt genoemd. Bij dit proces worden kleine beschadigingen in het epitheel door snelle celmigratie gerepareerd. Dit fenomeen is nog niet eerder met de slokdarm in verband gebracht. Bij de patienten die hun medicijnen hadden doorgebruikt werden ook expressie-veranderingen gevonden van enkele van deze genen maar minder in aantal en in mindere mate dan bij de eerste groep.

Hoofdstuk 9 laat de resultaten zien van een soortgelijk onderzoek, hier werden echter biopten uit het duodenum vergeleken tussen de drie groepen van tien mensen. Ook hier werden genen gevonden die anders tot expressie kwamen dan bij controles, eveneens vooral in de groep die twee weken geen medicatie gebruikt had. De genen die werden gevonden hadden taken in de opname en transport van vetzuren, resynthese van triglyceriden, formatie van chylomicronen en exocytose hiervan. Er werden aanwijzingen gevonden dat deze genexpressieveranderingen zouden kunnen zorgen voor een groter aandeel van apolipoproteine A-IV (ApoA-IV) in chylomicronen, dit is een signaalstof die zorgt voor cholecystokinine(CCK)-release uit neuro-endocriene cellen. Dit is een aanwijzing dat de invloed van het duodenum op de perceptie van zure reflux gemedieerd zou kunnen worden via ApoA-IV en CCK.

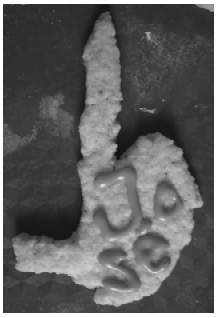
Dankwoord

Prof. dr. M Samsom
Beste Melvin, ondanks mijn gebrek aan ervaring nodigde je mij uit om het onderzoek dat geleid heeft tot dit proefschrift voort te zetten. Onze manier van overleggen – zelden gepland, nooit zonder beslissingen, altijd ruimte voor associatieve bespiegeling en een breder perspectief – impliceert een vertrouwen dat me heeft verbaasd en verheugd.

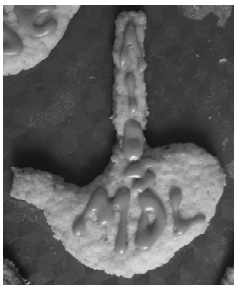
Prof. dr. A.J.P.M. Smout
Beste André, van jou heb ik geleerd hoe je je resultaten opschrijft. Zonder jouw strepende pen was dit proefschrift stukken minder leesbaar geweest. Ik dank je daarvoor en hoop nog vele pagina's met je op te stellen.



Dr. M.A. van Herwaarden
Beste Margot, jouw klinische blik en ervaring als reflux-onderzoeker zijn essentieel geweest bij het typeren van de patiëntengroepen waaruit de resultaten van de beschreven studies zijn ontstaan. Door jou voelde ik me direct thuis bij de MDL, mijn dank daarvoor is groot.



Dr. J.J.M. ter Linde
Beste José, jij behandelde mij vanaf het begin hebt als een volwaardige onderzoekspartner, in het lab en op papier. De reserve waarmee je mijn dikwijls voorbarige triomfen ontving heeft me enorm geamuseerd. Voor je hulp bij het behalen ervan dank ik je.



Onderzoekers en AIOS en staf van de maag-darm-leverziekten
Allemaal enorm bedankt, in het bijzonder Ofke en Marian, die me dikwijls uit een aanval van chagrijn trokken, Niels en Astrid voor de onvergetelijke Utrechtse bloed- en DNA-dagen, Joyce van Kuik voor het leren werken met de LDA's, Jac, Joke en Ria voor de tijd die altijd gemaakt kon worden voor het verzamelen van kandidaten, en alle anderen voor de rest van de hulp, het is niet te overzien!

Collega's van de divisie interne geneeskunde & dermatologie
Dank voor de extra tijd die me gegund werd om te schrijven, precies op het goede moment.

Curriculum vitae

Durk Rimmer de Vries werd op 27 april 1978 geboren in Gouda. Hij groeide op in Boskoop en maakte zijn eerste plannen om dokter te worden op R.K. Jenaplanschool de Zevensprong. Na het afronden van het atheneum aan het Groene Hart Lyceum in Alphen aan den Rijn in 1996 studeerde hij geneeskunde aan de Universiteit Utrecht. Tijdens deze studie werkte hij als verpleeghulp in diverse zorginstellingen en begeleidde werkgroepen als student-assistent. In 2003 vervulde hij een co-assistentschap keel-, neus- en oorheelkunde in het Massachusetts Eye and Ear Infirmary in Boston. Van december 2003 tot januari 2005 werkte hij als arts-assistent in het Zuwe Hofpoort Ziekenhuis in Woerden. Van januari 2005 tot december 2007 verrichtte hij promotieonderzoek bij prof.dr. Samsom en prof.dr. Smout op de afdeling maag-darm-leverziekten van het Universitair Medisch Centrum Utrecht. Tijdens deze periode gaf hij tevens onderwijs aan studenten geneeskunde en daarbuiten verrichtte hij samen met M. Veenhuizen onderzoek naar gastro-intestinale klachten bij Ehlers-Danlos-patiënten, en bakte hij vele appeltaarten. In 2007 werd hij aangesteld als arts in opleiding tot specialist (AIOS) in de maag-darm-leverziekten in de regio Utrecht. Thans doorloopt hij de vooropleiding bij de divisie interne geneeskunde in het UMCU.